

REGULATION OF MAST CELL FUNCTION BY TIM-1 AND TIM-3 SIGNALING

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The T cell (or transmembrane) immunoglobulin and mucin domain (TIM) family proteins have attracted significant attention as novel immune regulators. Tim-3 is expressed on chronically stimulated, often dysfunctional, T cells. Antibodies to Tim-3 can enhance anti-viral and anti-tumor immune responses. It is also constitutively expressed by mast cells, NK cells and specific subsets of macrophages and dendritic cells. There is ample evidence for a positive role for Tim-3 in these latter cell types, which is at odds with the model of Tim-3 as an inhibitory molecule on T cells. On the other hand, polymorphisms in the *TIM-1* gene, particularly in the mucin domain, have been associated with atopy and allergic diseases in mice and human. Genetic- and antibody-mediated studies revealed that Tim-1 functions as a positive regulator of Th2 responses, while certain antibodies to Tim-1 can exacerbate or reduce allergic lung inflammation. Tim-1 can also positively regulate the function of B cells, NKT cells, dendritic cells and mast cells. At this point, little is known about the molecular mechanisms by which Tim-1 and Tim-3 regulate the function of T cells or other cell types.

We have focused on defining the effects of Tim-1 and Tim-3 ligation on mast cell activation, since these cells constitutively express both proteins and are activated through an ITAM-containing receptor for IgE (Fc ϵ RI), using signaling pathways analogous to those in T cells. Employing a variety of gain- and loss-of-function approaches, we find that Tim-3 acts at a receptor-proximal point to enhance Lyn kinase-dependent signaling pathways that modulate both immediate-phase degranulation and late-phase cytokine production downstream of Fc ϵ RI ligation. Using a Tim-1 mouse model lacking the mucin domain (Tim-1 ^{Δ mucin}), we show for the first time

that the polymorphic Tim-1 mucin region is dispensable for normal mast cell activation. We further show that Tim-4 cross-linking of Tim-1 enhances select signaling pathways downstream of FcεRI in mast cells, including mTOR-dependent signaling, leading to increased cytokine production but without affecting degranulation. Thus, Tim-1 and Tim-3 are promising targets in development of therapeutics against mast cell-mediated allergic and autoimmune diseases.

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PREFACE

I would like to thank my mentor Dr. Lawrence Kane for believing in me and giving me the intellectual freedom to explore my thesis questions and develop as a scientist. I would like to thank my thesis committee members for their guidance and encouragement throughout my graduate career. I would also like to thank all of the Kane lab members past and present. I am forever grateful to my family for supporting me in life and in my decision to pursue a career in science.

1.0 INTRODUCTION

1.1 MAST CELLS

1.1.1 Mast cell origin and tissue distribution

Mast cells have been traditionally recognized as innate cells with central roles in atopic disorders and anaphylaxis. Their localization at the host-environment interface allows them to rapidly mount a response against invading pathogens and foreign substances. Mast cells develop from CD34⁺/CD117⁺ pluripotent progenitor cells originating from the bone marrow. Progression of mast cells through maturity is dependent on stem cell factor (SCF) produced by stromal cells. c-Kit (CD117), the receptor for SCF expressed on hematopoietic stem cells, is retained on mast cells throughout development, while its expression is down-regulated on other bone marrow cells during differentiation. In both mouse and human, mast cells progenitors (MCps) exit the bone marrow and subsequently migrate into peripheral tissues where they mature and become terminally differentiated mast cells, depending on the influence of cytokines and chemokines within the surrounding environment. Mast cells are distributed in a variety of tissues, particularly at the interface of the host and the external environment, i.e. sites such as skin, gastrointestinal (GI) tract, and the respiratory mucosa (1-4). Mast cells also reside in connective tissue surrounding blood vessels, nerves, hair follicles, and mucus glands (1).

The migration of MCps occurs in a tissue-specific manner. MCps are constitutively found in large number in the small intestine and rely on binding of $\alpha 4\beta 7$ integrins on mast cells to either

mucosal addressin cell adhesion molecule-1 (MAdCAM-1) or vascular cell adhesion molecule-1 (VCAM-1) expressed on the endothelium for tissue homing (5), (6). In addition, CXC chemokine receptor 2 (CXCR2) has also been associated with migration of mast cells into the small intestine. Under homeostatic conditions, MCps do not accumulate in the lung but they can be recruited during chronic allergen-induced inflammation (7). This recruitment is promoted by interactions between $\alpha 4\beta 7$ or $\alpha 4\beta 1$ integrins with VCAM-1 and CXCR2 on the endothelium, as well as by the chemokine C-C motif receptor 2 (CCR2)/C-C motif ligand 2 (CCL2) axis (8). Homing of MCps to the peritoneal cavity and certain regions of the skin has also been attributed to integrins (9). Finally, mast cells express a multitude of chemokine receptors, making them responsive to chemokines such as monocyte chemotactic protein-1 (MCP-1), CCL5, macrophage inflammatory protein-1 alpha (MIP1 α) at the site of inflammation (10).

Mast cells were first described in 1878 by Paul Ehrlich as metachromatic, granulated cells that stained positive for the basic aniline dye. The content of these granules, together with the tissue location, functional, and structural characteristics helped define two subtypes of mature mast cells: mucosal mast cells (MMCs) and connective tissue mast cells (CTMCs) (11). In mouse, MMCs reside in the mucosal epithelium of the lung and GI tract, and are characterized by the chymases mast cell protease (MCP)-1 and MCP-2. CTMCs are found primarily in the skin, intestinal mucosa and peritoneum, and contain the chymase MCP-4, the tryptases MCP-5 and MCP-6, as well as carboxypeptidase A. Upon activation, MMCs secrete a small amount of histamine and large quantity of cysteinyl leukotrienes, while CTMCs release larger amounts of histamine and prostaglandin D2 (PGD₂) (12). Furthermore, MMCs have been termed T-cell dependent mast cells, as they are absent in athymic nude mice (13). Using mouse bone marrow-derived mast cells (BMMCs), studies were conducted to identify the growth factors essential for

mast cell growth and development. IL-3 in particular was found to be responsible for mast cell survival, maturation and development of mucosal mast cells (14). On the other hand, SCF alone was sufficient to support mast cell survival and development *in vitro*, although mast cell culture can be enriched with a combination of SCF and IL-3 (15).

Human mast cells are also divided into two subsets based on their protease content. The tryptase/chymase (MC_{TC}) subset containing tryptases, chymases, and carboxypeptidases are found in skin, lymph nodes, lung, and gut mucosa. The MC_T reside in the intestinal and pulmonary mucosa, and stores only tryptases (16-18). In addition, MC_{TC} but not MC_T express the receptor for complement C5a (19).

1.1.2 Mast cell biology

Similar to other cell types of the immune system, activation of mast cells results in rapid generation and release of pro-inflammatory mediators. The immediate mast cell response is degranulation, which is characterized by expulsion of preformed cytoplasmic granule contents by exocytosis. Degranulation is a highly regulated process that requires assembly of granule-containing vesicles and fusion with the plasma membrane (20). Mast cell granules are rich in pre-formed bioactive amines, most notably histamine, which induces bronchoconstriction, vasodilation, capillary permeability, smooth muscle contraction, mucus secretion and mucosal edema, all of which are associated with allergic or inflammatory reactions (21, 22).

Mast cells also contain a significant amount of proteases, mainly tryptases and chymases which, as discussed above, have been used to define mast cell phenotypes depending on their abundance (23). Mast cell proteases have been implicated in arthritis, allergic airway

inflammation, innate immune defense, glomerulonephritis, and abdominal aortic aneurism formation (24-27). Conversely, mast cell proteases can also have immunomodulatory role in allergic reactions. Specifically, β -tryptase released by activated mast cells can limit the extent of allergic inflammation by cleaving IgE off its receptor (28). Mast cell granules also contain several species of lysosomal enzymes, one of which is the ubiquitous β -hexosaminidase present in all mast cell types and species. Thus, release of β -hexosaminidase is used to quantify mast cell degranulation (22). It is worth noting that mast cell granules also possess pre-formed TNF α , which can be rapidly released upon activation (29). Mast cell granules contain broad-spectrum anti-microbial peptides against bacteria, enveloped viruses, fungi and protozoa that accumulate at sites of infection. Furthermore, the human anti-microbial peptide cathelicidin can also act as a chemoattractant for neutrophils (30).

Aside from pre-formed mediators, activated mast cells can rapidly synthesize eicosanoids, a collection of inflammatory lipids that are generated from membrane phospholipids upon activation of phospholipase A₂. These species include leukotrienes (LT), prostaglandin (PG), and platelet-activating-factor (PAF) with functions in vascular permeability, bronchoconstriction in lung, recruitment of immune effector cells, as well as wheal-and-flare responses associated with allergic skin reactions (31).

While degranulation occurs within minutes of IgE crosslinking, *de novo* synthesis of cytokines and chemokines, resulting from enhanced transcriptional activation and gene expression, characterizes the late-phase response of mast cell activation. This leads to generation of a variety of pro- and anti-inflammatory cytokines, including TGF- β , TNF α , GM-CSF, IFN γ , IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-18, IL-13, chemokines such as CCL2, CCL3, CCL4, CCL5, CXCL8, growth factors including SCF, FGF, VEGF, and angiogenin (12, 32). These mediators

can have consequential effects on the target and surrounding tissues, resulting in numerous cellular responses such as allergic inflammation, autoimmunity, tumorigenesis and angiogenesis.

1.1.3 Mast cells in allergy

Perhaps the best-known role of mast cells is their promotion of allergic reactions that occur when the immune system mounts an overt response against otherwise “innocuous” antigens. In allergic reactions such as asthma, mast cells are responsible for both the acute and late phase reactions, through instantaneous release of pre-formed and *de novo* synthesized mediators. Specifically, the release of histamine, PGD₂, and leukotriene C₄ contribute to bronchoconstriction, mucus secretion, and mucosal edema (33). In the late phase, mast cell-derived cytokines recruit eosinophils, basophils, and T cells to the site of inflammation, and subsequently orchestrate tissue remodeling and fibrosis, as commonly seen in asthma, allergic rhinitis and atopic dermatitis (34-36).

1.1.4 Mast cells in autoimmune diseases

Since mast cells can maintain persistent inflammation and recruit immune cells to the site of inflammation, it is possible that this inflammatory environment becomes favorable to self-reactive lymphocytes that can cooperate with mast cells to inflict tissue damage. Mast cells are associated with several autoimmune diseases, including multiple sclerosis (MS) and rheumatoid arthritis (RA). Specifically, mast cell numbers and distribution have been correlated with development of MS (37-41). Mast cell stabilizing drugs such as sodium cromoglycate were able to relieve the severity of allergic encephalitis (EAE), an experimental model of MS (42). In RA, mast cells are

found to accumulate in the synovial tissues and fluids of patients and can locally release inflammatory mediators (43). The complement pathway, Fc receptor binding, IL-1 and TNF have all been associated with RA development (44-47).

1.1.5 Mast cells in innate immunity

Because of their location at barrier surfaces, mast cells are among the first responders of the immune system to foreign pathogens, antigens, and toxin. As such, mast cells express a whole host of surface receptors to detect potentially harmful signals, through which they can induce rapid release of pre-formed and *de novo* synthesized mediators. Mechanisms of recognition range from pathogen-associated molecular pattern (PAMP) receptors such as Toll-like receptors (TLRs), complement receptors, immunoglobulin receptors, as well as receptors that recognize damage-associated peptides produced by infected or injured cells (33). The responses generated by activated mast cells against bacteria depend on the type of PAMPs present. For example, TLR4 binding to LPS from gram-negative bacteria, lipid A or fibrinogen results in production of pro-inflammatory cytokines like TNF- α , IL-1 β and IL-6, without triggering degranulation. On the other hand, TLR2 recognizes peptidoglycans from gram negative bacteria and mycobacteria, leading to both cytokine production and degranulation (48, 49). In addition, mast cells secrete anti-microbial peptides such as cathelicidins and defensins, reactive oxygen species, and can also directly phagocytose bacteria (30, 50, 51).

The role of mast cells in viral infection is less clear. Mast cells can be infected by human immunodeficiency virus (HIV), dengue virus, Influenza A virus, cytomegalovirus and adenoviruses. Virally infected mast cells produce multiple soluble mediators, including IL-1 β , IL-6, CCL3, CCL4, CCL5 and CCL8, all of which serve to recruit NK and NK T cells to site of

infection (52-55). Mast cells can also directly inactivate viruses by secretion of anti-microbial peptides (56).

In parasitic infection, mast cell mediators can regulate tissue-derived cytokines like IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), which are critical for optimal Th2 responses and worm expulsion (57). Thus, in these types of infections mast cells contribute by producing a favorable environment for parasite containment and clearance.

1.1.6 Mast cells in adaptive immunity

While less robust than dendritic cells (DCs), studies have shown that mast cells can present antigens via MHC class I and II complexes (51, 58, 59). Activation of mast cells through TLR7 triggers the release of IL-1 β and TNF, leading to DC recruitment to lymph nodes and induction of T cell cytotoxic responses (60, 61). Mast cells can also release exosomes containing co-stimulatory molecules to directly promote maturation and function of DCs (62). Furthermore, mast cells have been shown to form conjugates with T cells and can directly activate T cells through TNF production (63, 64). Conversely, mast cells can act to limit the magnitude and duration of an immune response through secretion of immunomodulatory cytokines like IL-10 and TGF- β (35, 65, 66).

Given their broad pro-inflammatory potential, it is somewhat unexpected that mast cells also participate in immune tolerance. Specifically, mast cells can promote allograft tolerance through secretion of IL-10 and TGF- β , and through cytokine-mediated interactions with regulatory T cells in skin and heart transplants. Mast cell-derived IL-2 was recently shown to support an anti-inflammatory ratio of effector-to-regulatory T cells in a model of contact dermatitis (67, 68). Moreover, OX40L-expressing mast cells can interact with OX40-presenting regulatory T cells, an

interaction that serves to inhibit mast cell degranulation without affecting cytokine production (69-71). Finally, mast cell histamine and lipid mediators also have a role in immune suppression. For instance, histamine binding to histamine receptor 2 (HR2) inhibits the inflammatory response to UVB radiation, while PGE2 can trigger IL-10 production by DCs and inhibit DC maturation (72-74).

1.1.7 Mast cells in tissue repair, angiogenesis, and cardiovascular disease

In addition to promoting immune responses, mast cells participate in the maintenance of tissue homeostasis (75). In fact, mast cells participate in all steps of tissue repair, ranging from the initial inflammation to extracellular matrix remodeling. The mast cells mediators nerve growth factor (NGF), PDGF, VEGF and fibroblast growth factor-2 (FGF-2) induce epithelial cell and fibroblast proliferation (76). They activate platelets and promote both extravascular deposition of fibrin and the appropriate perfusion and nutrition necessary for repair (77-80). Mast cells also produce IL-1, IL-4, IL-6 and FGF to induce myofibroblasts, which are important for wound healing (81). Mast cell histamine, IL-1, IL-6, TGF β and PDGF can all contribute to osteoclast recruitment and development and consequently to bone remodeling (82).

The proximity of mast cells to blood vessels and their release of angiogenic factors, including angiotensin-1, FGF-2, VEGF, IL-8, TNF, histamine and heparin implicates a role for mast cells in angiogenesis (83). Specifically, these mediators can break down and rebuild extracellular matrix (ECM), induce proliferation of endothelial cells and promote the formation of new vessels (84). While these factors are not produced exclusively by mast cells, further support for a function of mast cells in angiogenesis is based on the observation that mast cell-specific

proteases like MCP-4, tryptase, histamine and heparin can promote vascular tube formation, particularly in the tumor microenvironment (12).

Cardiac mast cells are present in the coronary arteries during spasm and accumulate in atherosclerotic plaques (85). Furthermore, chymase and tryptase released by mast cells can interfere with cholesterol efflux by macrophages leading to formation of foam cells that contributes to atheroma (43, 86, 87).

1.1.8 Mast cells in cancer

Mast cells appear to be involved in both cancer promotion and anti-tumor immunity, although the clinical relevance of mast cell/tumor interactions remains to be determined. Mast cells secrete large quantities of pro-angiogenic factors such as angiopoietin-1, TNF, VEGF and PDGF, which promote ECM degradation and vascular formation (88). Mast cells can also release immunosuppressive cytokines like IL-10 and TNF- α , which contribute to tumor growth. Mast cells also secrete metalloprotease (MMP)-2 and MMP-9 and heparin to promote metastasis (89-91). On the other hand, mast cell-derived TNF- α , IL-1 and IL-6, were shown to suppress melanoma growth (92, 93). Finally, eosinophil recruitment and survival is supported by mast cell tryptase and IL-5, which leads to tumor regression (88).

1.2 MAST CELL SIGNALING

1.2.1 Early FcεRI signaling

While mast cells express a multiplicity of surface receptors that can trigger cellular responses, much focus has been placed on understanding the signaling mechanisms downstream of antigen-IgE-dependent aggregation of the high affinity IgE receptor (FcεRI). FcεRI belongs to the immunoglobulin receptor superfamily and is expressed as a tetrameric complex composed of the IgE-binding α chain, which is unique to this receptor, the membrane-tetraspanning β chain and a disulfide-linked γ homodimer, shared among other immune receptors (94-98). (99, 100)The β chain serves as a signal amplifier while the γ chains impart signaling competence for the receptor (99, 100). Upon antigen cross-linking of IgE-bound FcεRI, receptor aggregation leads to transphosphorylation of the immunotyrosine-based activation motifs (ITAMs) of the β and γ chains by the Src family kinase (SFK) Lyn, which constitutively associates with the β chain (101, 102). Additional Lyn kinase is then recruited to the phosphorylated β chains, while the phosphorylated γ chains serve as docking sites for the spleen tyrosine kinase (Syk) (102-104) (Figure 1.1). While the β and γ chains are generally viewed as positive regulators of FcεRI signaling, recent studies provided evidence suggesting that they also negatively regulate mast cell activation. The FcεRI β ITAM possesses a non-canonical tyrosine residue in between two canonical tyrosines found in conventional ITAMs. The loss of the canonical tyrosine 219 (Y219) resulted in marked reduction of Lyn binding and partially inhibited degranulation and cytokine production, suggesting that this is the primary recruitment site of Lyn and mediator of positive signaling (105, 106). In contrast, mutation of the noncanonical Y225 unexpectedly increased NF-

κ B activation and cytokine production without affecting degranulation, together with diminished phosphorylation of the SH2-containing phosphatases (SHP)-1 and SHP2 and the SH2-containing inositol phosphatase (105, 107, 108). Finally, Fc ϵ RI β can recruit positive or negative regulators, depending on the strength of the stimulus (109). Fc ϵ RI γ has also been found to mediate negative regulation of signaling to terminate cellular activation. Studies using Fc α RI, a receptor that also uses Fc ϵ RI γ for activation showed that cross-linking of Fc α RI can inhibit Fc ϵ RI-dependent mast cell degranulation in a process that involves association of the γ ITAMs with the phosphatase SHP-1 (110). These studies have modified the view that assigned solely positive functions to Fc ϵ RI β and γ chains, and hence suggest a more complex relationship between how Fc ϵ RI regulates the transmission of positive and negative signals.

Antigen-mediated cross-linking of Fc ϵ RI is required for robust mast cell activation. This is due to the increased surface receptor complex expression and stabilization upon IgE binding (111). There is evidence that IgE can bind to Fc ϵ RI and promote mast cell survival without inducing antigen cross-linking (112). These results provide a potential explanation to the contribution of IgE as a signal amplifier of allergic reactions.

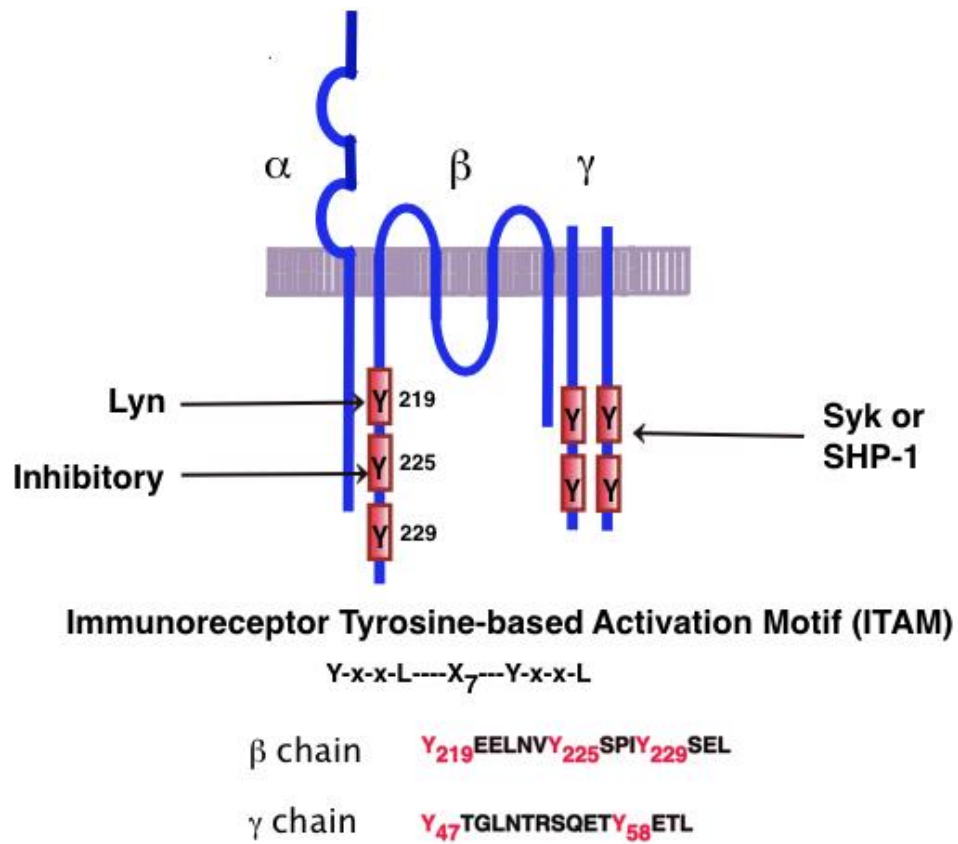


Figure 1-1: The FcεRI complex. Schematic representation of the FcεRI complex. Tyrosines as reported binding sites for the positive and negative signaling molecules within the ITAM motifs are indicated in red.

1.2.2 Tyrosine kinases in FcεRI proximal signaling

Lyn is the predominant SFK in mast cells and is estimated to be at least 20-fold more abundant than Fyn and Hck (113, 114). Lyn association with FcεRI is a pre-requisite for antigen receptor phosphorylation and several models have been proposed to explain the initial Lyn-mediated FcεRI activation. Lyn, via its SH2 domain, constitutively associates with basally phosphorylated FcεRI β, while the SH4-containing domain of Lyn can also bind to FcεRI β (115). Another, more debatable, model suggests both constitutive and stimulation-dependent interactions between FcεRI and Lyn in the cholesterol- and phospholipid-rich lipid rafts of the membrane. This model proposes that a small fraction of Lyn and FcεRI are constitutively present in these lipid raft microdomains, where a lipid-rich environment can help stabilize the FcεRIβ-Lyn interaction and the receptor signaling complex after antigen stimulation (116, 117).

Initial studies using induction of passive systemic anaphylaxis (PSA), an indicator of mast cell function, demonstrated that Lyn-deficient mice had diminished PSA, which correlated with decreased phosphorylation of Syk and other downstream molecules (118). These observations supported a positive role for Lyn in the initiation of FcεRI signaling. Nevertheless, studies using Lyn-deficient mast cells reported either unchanged or enhanced degranulation *in vitro* (119, 120). These results raised the possibility that Lyn may also negatively regulate FcεRI activation. It is now known that Lyn is required for phosphorylation of the C-terminal Src kinase (Csk)-binding protein (Cbp), a negative regulator of SFKs through its recruitment of Csk. Indeed, Fyn activity in mast cells is enhanced in the absence of Lyn, which partially explains the increase in mast cell degranulation (113). Furthermore, there is defective phosphorylation of the phosphatases SHP-1 and SHIP in Lyn-deficient mast cells (109, 120). In addition, published findings show that stimulus

strength can dictate whether FcεRIβ takes on a negative or positive regulatory role, and that Lyn association with FcεRIβ is increased under high intensity stimulation. Thus, the function of Lyn in FcεRI activation is clearly complex and interpreting the effects of modulating FcεRI signaling requires taking into consideration how receptor intensity affects Lyn and in turns mast cell effector function.

Studies using Lyn-deficient mast cells have revealed Fyn kinase as a positive regulator of the complementary pathway downstream of FcεRI activation (113). Fyn-deficiency is associated with diminished mast cell degranulation, which has been attributed to decreased phosphorylation of Grb2-associated binding protein 2 (Gab2) and subsequent activation of PI3K (113, 119, 121, 122). Specifically, absence of Fyn led to a significant reduction in phosphatidylinositol-3,4,5-triphosphate (PIP₃), a product of PI3K, and also reduced degranulation. Fyn has also been shown to promote calcium influx by either phosphorylating the plasma membrane calcium channel transient receptor potential channel (TRPC) or activating the sphingosine-1-phosphate kinase 2 (SphK2) (123, 124). In addition, Fyn can affect degranulation in calcium-independent ways. Fyn is involved in chemotaxis, vesicular trafficking, cytoskeleton reorganization and regulation of microtubule formation (125, 126). Thus, Fyn augments many aspects of FcεRI signaling required for mast cell activation and effector function.

Syk is indispensable for FcεRI signal propagation and effector responses, as Syk-deficient mast cells do not exhibit degranulation and cytokine production (127, 128). Furthermore, Syk-deficient mast cells are unable to activate NF-κB or NFAT (129, 130). Binding of Syk, via its Src-homology 2 (SH2) domains, to phosphorylated FcεRI γ ITAMs results in conformational changes that disrupts the inhibitory interaction between COOH-terminal/SH2-interdomain leading to Syk activation and increased enzyme activity (103, 129). Tyrosine phosphorylation of Syk upon

receptor aggregation is mostly due to autophosphorylation, with limited contribution from other tyrosine kinases like Lyn (131, 132). Phosphorylation mapping with *in vitro* kinase reactions identified ten tyrosines that are autophosphorylated (133). These phosphorylated Syk tyrosines mediate either positive or negative regulatory roles in FcεRI signaling. For instance, mutation of Syk Y317 in RBL-2H3 basophilic cells enhanced PLCγ phosphorylation and increased degranulation, while mutations of Y519 and Y520 led to decreased degranulation (134-136). Furthermore, phosphorylation of Syk Y346 is entirely dependent on Lyn, while tyrosines 317, 342, 519, and 520 are regulated by both auto-phosphorylation and trans-phosphorylation by Lyn (131). The activation state of Syk is important, as these tyrosine phosphorylation events provides binding sites for both positive and negative regulators of downstream signaling. The c-Cbl protein, which binds to Y317, mediates ubiquitination of FcεRI and Syk after antigen activation, likely to dampen antigen receptor signaling (137). On the other hand, Y342 is required for binding and Syk-mediated phosphorylation of SLP-76, LAT and PLCγ2, which lead to calcium mobilization and degranulation (Zhang, 2002). Finally, mutations in the conserved tyrosines 624 and 625 decrease Syk binding to phosphorylated ITAMs, leading to decreased kinase activity and reduced degranulation, MAPK kinases activation and NFAT and NF-κB activation (129, 133, 138).

The SFKs Fgr, Hck and Src are also expressed in mast cells, but their precise roles are less clear (99). Fgr is able to phosphorylate PLD₂ and likely contributes to degranulation in that manner (139). Src was reported to bind PKCδ, a kinase that phosphorylates FcεRIγ, although the effect of this interaction is not known (140). Studies using Hck-deficient mast cells showed that Hck is a positive regulator of mast cell degranulation and cytokine production, and can negatively regulate Lyn (114). Collectively, these findings suggest a relatively modest, non-dominant role for these kinases in mast cell function.

The cytosolic Tec family kinases Btk, Itk, Rlk and Tec are expressed in mast cells but only Btk and Itk have been shown thus far to have significant effects on mast cell activation (141). Btk is activated by Src-dependent tyrosine phosphorylation within its activation loop, which has been shown to depend on Lyn in mast cells (142, 143). In antigen activated mast cells, Btk translocates to the membrane via binding of its PH domain to membrane-associated PIP₃, where it associates with PKC β 1 and likely phosphorylate PLC γ 1 and PLC γ 2, thereby contributing to degranulation and cytokine production (143-145). The relative contribution of Itk in mast cells has also been examined, with conflicting results. Mouse bone marrow-derived mast cells (BMMCs) lacking Itk show no obvious defects in antigen-induced degranulation. However, IL-13 and TNF- α release is enhanced and is associated with increased nuclear NFAT activity (146). On the other hand, in the same study, transfer of Itk^{-/-} BMMCs to mast cell-deficient mice was still able to rescue histamine release in vivo, suggesting that the defective responses are not due to absence of Itk in mast cells (146).

1.2.3 Fc ϵ RI signaling in degranulation and cytokine production

Antigen cross-linking of Fc ϵ RI induces phosphorylation of Syk, which mediates the phosphorylation of several adaptor proteins essential for further binding and recruitment of signaling molecules. Active Syk phosphorylates four tyrosine residues on the protein linker for activation of T cells (LAT) that serve as binding sites for Grb2/SOS, PLC γ , and Gads (135, 147, 148). Activation of LAT also recruits the key adaptor protein SH2 domain-containing leukocyte-specific phosphoprotein of 76kD (SLP-76), which is constitutively bound to Gads (149-152). This localization of SLP76 allows Syk to phosphorylate three N-terminal tyrosines of SLP-76 for

recruitment of Vav, Nck and Btk (153). More importantly, activation of LAT and SLP-76 results in recruitment of PLC γ 1 and PLC γ 2 to this multimolecular complex, leading to their phosphorylation by Btk and/or Syk (154-156). PLC γ 1 and PLC γ 2 activation leads to conversion of phosphatidylinositol 4,5-bisphosphate into inositol triphosphate (IP₃) and diacylglycerol (DAG). Generation of IP₃ drives mobilization of intracellular Ca²⁺ that is then sustained by influx of extracellular Ca²⁺, a critical event for mast cell degranulation (157). Downstream of the Ca²⁺ response, mast cell degranulation culminates in membrane fusion for granule exocytosis. This regulated event is mediated by the membrane fusion proteins soluble N-ethyl-maleimide-sensitive factor-attachment protein receptors (SNAREs) and the Rab GTPases (99, 158-161).

Phosphorylation of LAT also activates Ras/Raf, resulting in phosphorylation of the MAPKs extracellular signal-regulated kinases (ERK1,2,5), p38 and c-Jun N-terminal kinase (JNK) (162). These MAPKs go on to regulate activation of multiple transcription factors, including (AP)-1, NFAT and NF- κ B, and thus has a role in cytokine production (163-165). Furthermore, DAG generated by phosphorylated PLC γ phosphorylation activates protein kinase C (PKC) β and PKC ϵ in mast cells, contributing to Fos and Jun activation (166). In addition, ERK is involved in phosphorylation of PLA₂ and thus have a role in eicosanoid generation (167, 168). In addition to degranulation, PLC γ is also involved in cytokine production, primarily due to its ability to influence NFAT activity. The Ca²⁺ signal downstream of PLC γ 1 is required for calcineurin-mediated dephosphorylation and nuclear translocation of the transcriptional factor NFAT, and subsequent cytokine gene transcription and translation (169). Taken together, the signaling pathways discussed above form the Lyn/Syk/LAT/PLC γ axis that makes up the primary activation pathway downstream of Fc ϵ RI activation.

Due to its contribution to degranulation and cytokine production, Fyn kinase is responsible for mediating the complementary signaling pathway in antigen activated mast cells. This pathway does not involve LAT but rather activation of PI3K downstream of Gab2 phosphorylation (122). PI3K phosphorylates plasma-membrane-associated phosphoinositides at the 3' position, providing docking sites for PH domain-containing molecules such as PLC γ 1, PLC γ 2, Vav and Btk (149, 170). It has been proposed that PI3K might regulate Fc ϵ RI-mediated degranulation by recruiting Btk and PLC γ to the plasma membrane where PLC γ can be phosphorylated by Btk (163). On the other hand, PI3K can activate phospholipase D (PLD), which subsequently regulates the sphingosine kinase (SK)-S1P pathway involved in calcium mobilization in an IP₃-independent manner (171, 172). Similar to the LAT/PLC γ pathway, PI3K-mediated cytokine production may require the structurally similar non-T-cell activation linker (NTAL) as a transmembrane adaptor molecule (mainly to maintain, but not initiate, the calcium signal for optimal degranulation (149, 173-175).

The PI3K pathway has also been implicated in mast cell cytokine production since PI3K pharmacologic inhibition in mast cells and BMDCs with p110 δ mutation showed reduced cytokine production capacity (176). While the precise mechanisms leading to cytokine production are less clear compared to degranulation, PI3K activation can recruit the serine/threonine kinase 3-phosphoinositide-dependent protein kinase (PDK)-1 to phosphorylate Akt at the plasma membrane (177). In mast cells, Akt can promote NF- κ B activation by phosphorylating I κ B (178). Furthermore, through calcium mobilization, PI3K may be able to augment NFAT activation downstream of PLC γ . While the Fyn/Gab2/PI3K axis involves activation of different signaling molecules to regulate similar functional outcomes, it shares common downstream targets with the Lyn/Syk/LAT pathway, i.e. the activation of PLC γ and Vav, suggesting that these molecules may

be involved in integration and coordination of both pathways. Thus, how these pathways differentially regulate antigen-mediated activation of specific transcription factors for gene expression merits further study.

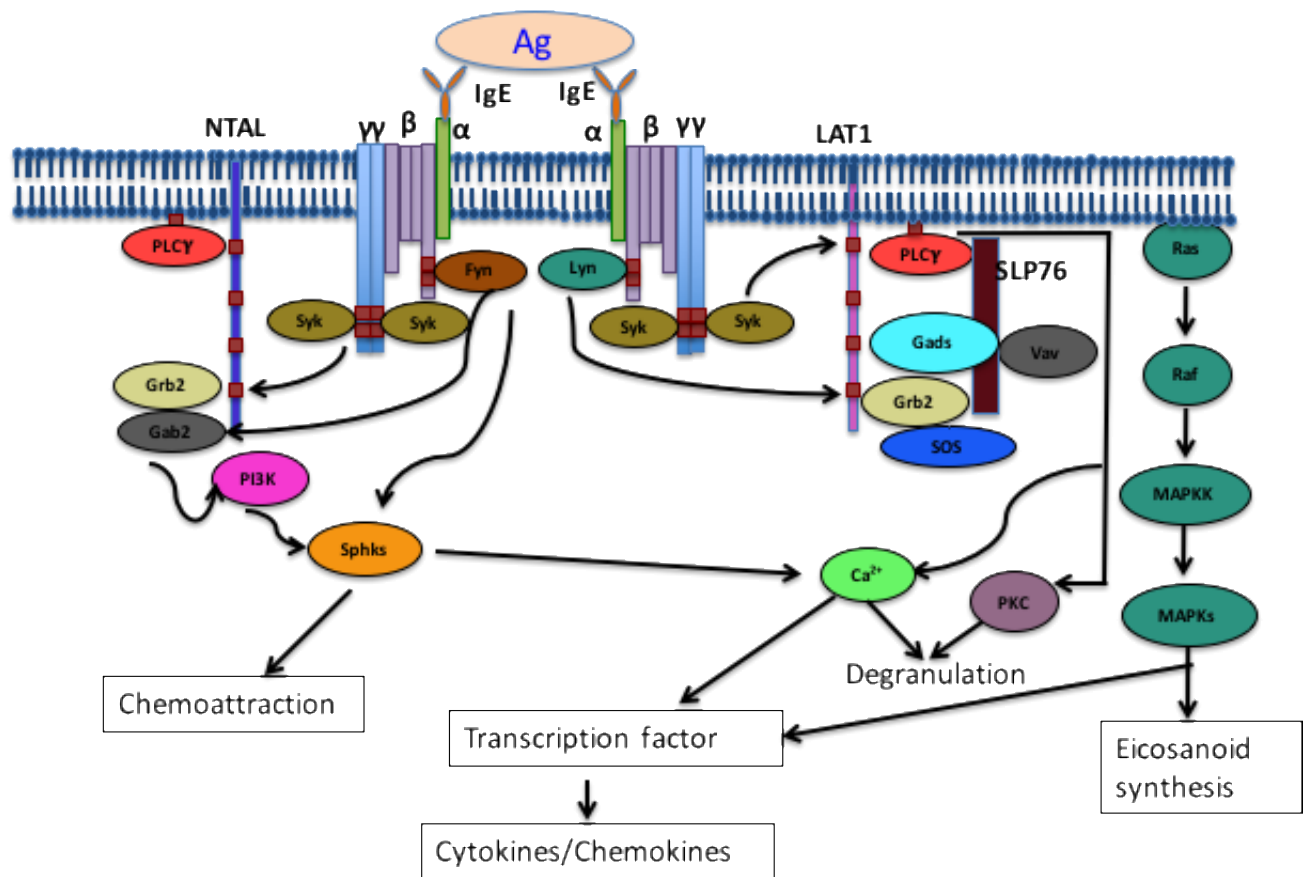


Figure 1-2: FcεRI-mediated signaling pathways.

1.2.4 Signaling by other mast cell surface receptors

The stem cell factor (SCF) receptor c-kit is crucial for mast cell growth, differentiation, survival and homing. Unlike FcεRI, c-kit is a single-chain receptor with intrinsic protein-tyrosine-kinase activity (179). Nevertheless, SCF induces similar signaling pathways as FcεRI signaling, including PLCγ1 activation, calcium mobilization, PI3K and MAPK activation, albeit with delayed kinetics and to a lower magnitude. Moreover, these signals are not sufficient to induce mast cell degranulation, but need to couple with the FcεRI signaling pathways to impact mast cell activation (180).

The ability to induce degranulation and cytokine release is not restricted to FcεRI and c-kit, but occurs upon ligand encounter by a wide range of mast cell receptors. A series of pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs), nod-like receptors (NLRs) and retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs) are expressed by mast cells. Specifically, stimulation of mast cell TLR culminated in the myeloid differentiation primary response gene (MyD88)-mediated activation of PI3K, MAPKs and NF-κB (181, 182).

Mast cells can be positively or negatively regulated by FcγRI and FcγRIII IgG or FcγRIIb respectively. These receptors allow mast cells to participate in humoral defense and antibody-induced pathologies (183). FcγRI and FcγRIII receptors belong to the same immunoglobulin receptor superfamily and share the same ITAM-containing γ subunit with FcεRI. As a result, aggregation of these receptors induce similar patterns of mediator release (184, 185). In contrast to FcγRI and FcγRIII, FcγRIIb contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs), and when aggregated with ITAM-containing receptors, can promote ITIM phosphorylation by

SFKs (186). Phosphorylated ITIMs can recruit phosphatases like SHIP-1 and SHIP-2, leading to suppression of signaling and downstream mediator release (187).

Mast cells also express receptors for the C3a and C5a complement components. The C3a receptor is a G-protein coupled receptor (GPCR) and upon activation promotes degranulation and production of MCP-1 and RANTES in human mast cells (188). Similarly, other GPCR receptors like adenosine 3, sphingosine 1 phosphate-2 (S1P₂), C-C Chemokine Receptor type 1 (CCR1), corticotropin-releasing hormone receptor (CRHR) and the beta-adrenoceptor are also present on mast cells and able to modulate antigen-mediated mediator release (189).

2.0 T CELL, TRANSMEMBRANE, IMMUNOGLOBULIN, MUCIN DOMAIN FAMILY OF PROTEINS

2.1 IDENTIFICATION OF *TIM* GENES

A complex interplay between environmental factors and genetic susceptibility is thought to underlie atopic diseases like asthma, atopic dermatitis and allergic rhinitis. Asthma susceptibility in particular has been linked to chromosome 5p23-35 because this region contains candidate genes like those encoding IL-12 p40, IL-9, the β -adrenergic receptor and the IL-4 cytokine cluster (IL-4, IL-5, and IL-13). To identify an asthma susceptibility gene from this complicated linked region, McIntire *et al* utilized congenic inbred mouse strains that differed only in discrete regions. They delineated a single region on chromosome 11, syntenic to human chromosome 5q, that conferred protection against T helper type 2 (Th2) responsiveness and airway hyperreactivity (AHR), a hallmark of asthma (190). This region is separate from the IL-4 cytokine gene cluster and was termed the *T cell and airway phenotype regulator (Tapr)*. Within this region was identified a family of genes that encode T cell membrane glycoproteins, a conserved immunoglobulin variable domain (IgV) and mucin domain. Due to their structures, these gene products were hence termed the T cell, immunoglobulin domain and mucin domain (Tim) proteins. There are 8 *Tim* genes on mouse chromosome 11 and 3 *TIM* genes on human chromosome 5q33. Murine Tim-1, 2, 3, 4 encode functional proteins, while murine Tims 5-8 appear to be pseudo-genes. Murine Tim-1 and Tim-2 are homologous to human TIM-1, and murine Tim-3 and Tim-4 bear strong sequence homology to human TIM-3 and TIM-4. Further sequence analysis revealed that mouse Tim-1 is

homologous to rat *Kim1*, or kidney injury molecule 1. In addition, mouse Tim-1 is homologous with human and monkey hepatitis A virus cellular receptor (hHAVcr1).

Around the same time that the *Tapr* region was cloned, Kuchroo and colleagues were searching for specific markers of Th1 T cells using an antibody (Ab)-generation approach. They identified a particular monoclonal antibody (mAb) that was able to recognize Th1 cell clones and *de novo*-generated Th1 T cells, but not naïve or Th2 T cells. Upon analysis of the antigen, it was determined to be murine Tim-3 (191). They also provided the first evidence that Tim-3 may function as an inhibitory molecule, since administration of a Tim-3 Ab exacerbated disease in the experimental autoimmune encephalomyelitis (EAE) model (191). Since the identification of the Tim family, others have reported their roles in immune regulation, including allergy, infection, autoimmune disease and cancer.

2.2 TIM PROTEIN STRUCTURE

The *TIM* genes encode type I immunoglobulin (Ig)-like domain proteins that also include a mucin domain with O-linked glycosylation and N-linked glycosylation sites close to the membrane, a single transmembrane domain and a cytoplasmic tail with tyrosine phosphorylation motif, except in Tim-4 (190). The crystal structures for the Ig-like domains of murine Tims 1-4 reveal that they are of the immunoglobulin V type (192, 193). This IgV domain is important for Tim-2 dimerization, while homophilic Tim-1: Tim-1 interactions require both the IgV and mucin domain (193). The IgV domain is composed of two anti-parallel β sheets, with short strands B, E, D in one (BED β -sheets) and A, G, F, C, C' and C'' β -strands in the other (GFC β -sheets). The most striking

feature is the deep pocket flanked by two hydrophobic loops that can extend into membranes. All Tim IgV domains contain six conserved cysteine residues, and the first and last of the cysteine residues bridge the β -sheets. The other four cysteines found in all Tim proteins form two disulfide bonds that create the folded CC' loop upward onto the GFC β -sheet. As a result, the tip of the CC' loop is parallel to the FG loop, and generate a distinct ligand binding pocket in Tim-1, Tim-3, and Tim-4, but not Tim-2 (193) (Figure 2-1). Identified by high resolution analysis of Tim-4 crystals, this unique pocket was shown to contain residues that coordinate with metal ions such as calcium, and thus has been termed the metal-ion-dependent ligand binding site (MILIBS). Phosphatidylserine (PS), a ligand of Tim-1, Tim-4 and Tim-3, is able to penetrate into the cavity of this loop, and requires metal ion coordination and conserved residues within this pocket for its binding (193). These conserved residues are only present in Tim proteins that bind PS but not Tim-2, which does not, and this interaction is specific for the L-stereoisomer of PS, thereby suggesting that the MILIBS pocket is specific for PS recognition and has specificity for phospholipid binding of the Tim proteins (193).

According to crystal structure models, the BC loop within the IgV domain of Tim-1, Tim-2 and Tim-3 is variable and contributes to interaction with the lipid bilayers of cell membranes, while PS interacts with the Tim proteins (192-194). In fact, there are seven amino acid differences between BALB/c and HBA Tim-3 that confer PS binding specificities and they lie within the BC loop (194). Therefore, variations within the IgV domains may contribute to ligand binding affinities among the Tim proteins. For instance, Cao *et al* demonstrated that, while the unique CC'-FG cleft is not required, glycosylation of Tim-3 IgV domain is needed for Tim-3 binding to the ligand galectin-9 (192). Furthermore, mutation of the CC'-FG cleft abolishes binding of Tim-3 to other cell types and transformed cell lines from different species (192). Collectively, these results

are consistent with a unique ligand binding function of the IgV domain and suggest the existence of multiple ligands for the TIM family of proteins (Figure 2-1).

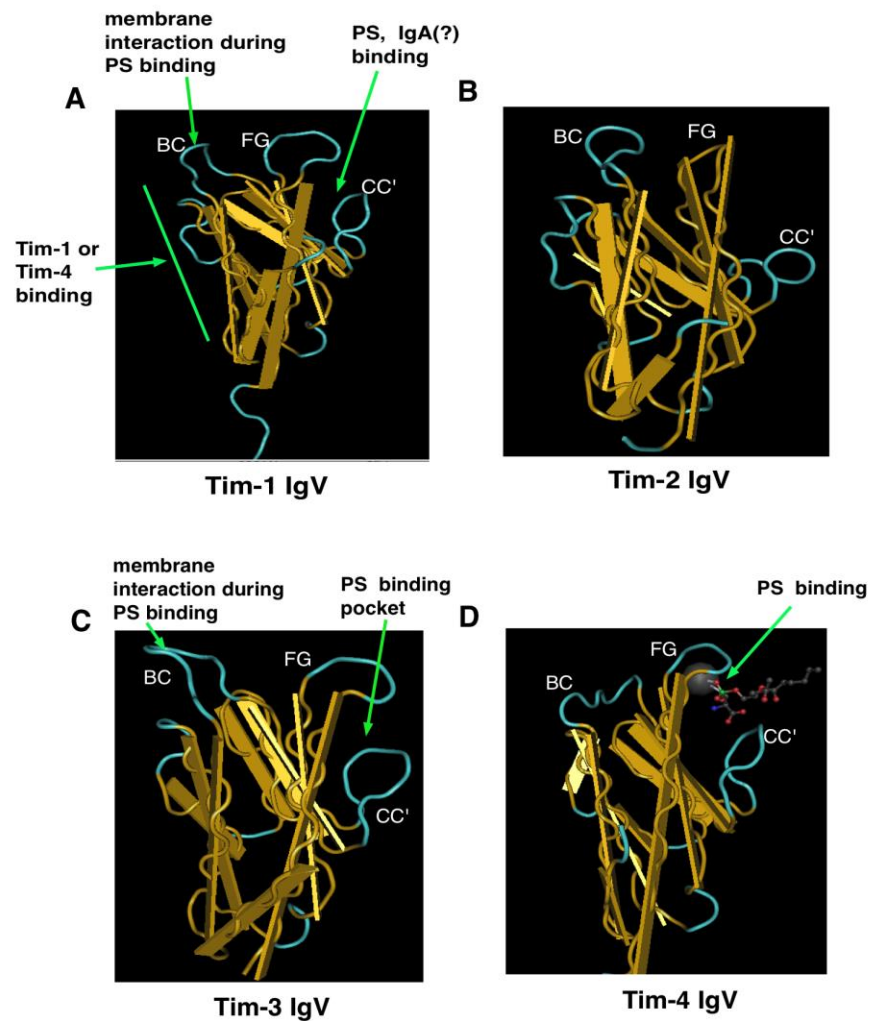


Figure 2-1: Structures of IgV domain and ligand binding sites. IgV domain structures of Tim-1(A), Tim-2 (B), Tim-3 (C) and Tim-4 (D). Reported ligand binding sites are indicated by arrows, based on crystal structures, ligand interaction, antibody blocking, and mutational studies. Structures are generated using Ch3D with structural information from the public database. Tim-1 PBD ID: 2OR8; Tim-2 PBD ID: 2OR7; Tim-3 PBD ID: 3KAA; Tim-4 PBD ID: 3BIB.

The size of the mucin domains varies considerably among members of the Tim family, but they are all rich in threonine, serine, and proline (190). Sequence analysis indicated that the Tim-1 mucin domain has multiple sites for O-linked glycosylation and the Ig domain has two putative N-linked glycosylation sites. Like Tim-1, Tim-2 also has two N-linked glycosylation sites and a heavily O-linked glycosylated mucin domain. Tim-3 has both N- and O-linked glycosylation sites in its IgV domain and much less glycosylation in the mucin domain, compared to Tim-1 and Tim-2. The IgV region of Tim-4 is not predicted to be glycosylated, but has multiple N-linked glycosylation sites in its mucin domain. Overall, the Tim proteins are expected to be highly glycosylated and bear structural resemblance to cell adhesion molecule mucosal addressin cell adhesion molecule-1 (MADCAM1). Of note, both murine and human TIM-4 have RGD integrin-binding motifs in their IgV domains that has been implicated in $\alpha v \beta 3$ integrin binding, to promote growth of non-small-cell-lung cancer tissue (192, 195, 196).

The cytoplasmic domain of Tim family proteins consists of 42-77aa and is the most conserved domain between human and mouse orthologs (191). There are two splice variants of TIM-1, the TIM1a that is mainly expressed by human liver and lacks the phosphorylation motif, and the TIM1b variant mainly expressed by human kidney cells and has two tyrosine residues including the highly conserved motif RAEDNIY (190, 197). Like Tim-1, Tim-2 also has an intracellular tyrosine phosphorylation motif, RTRCEDQVY, and is phosphorylated upon T cell activation (190, 198). Tim-3 cytoplasmic tail contains both tyrosine phosphorylation motif and an SH2 domain-binding motif (190). In contrast, Tim-4 does not have any conserved tyrosines, suggesting that it functions as a ligand rather than a signaling receptor (199). The structural variation in the Tim proteins suggest they have distinct intracellular signaling pathway upon ligand binding (Figure 2-2).

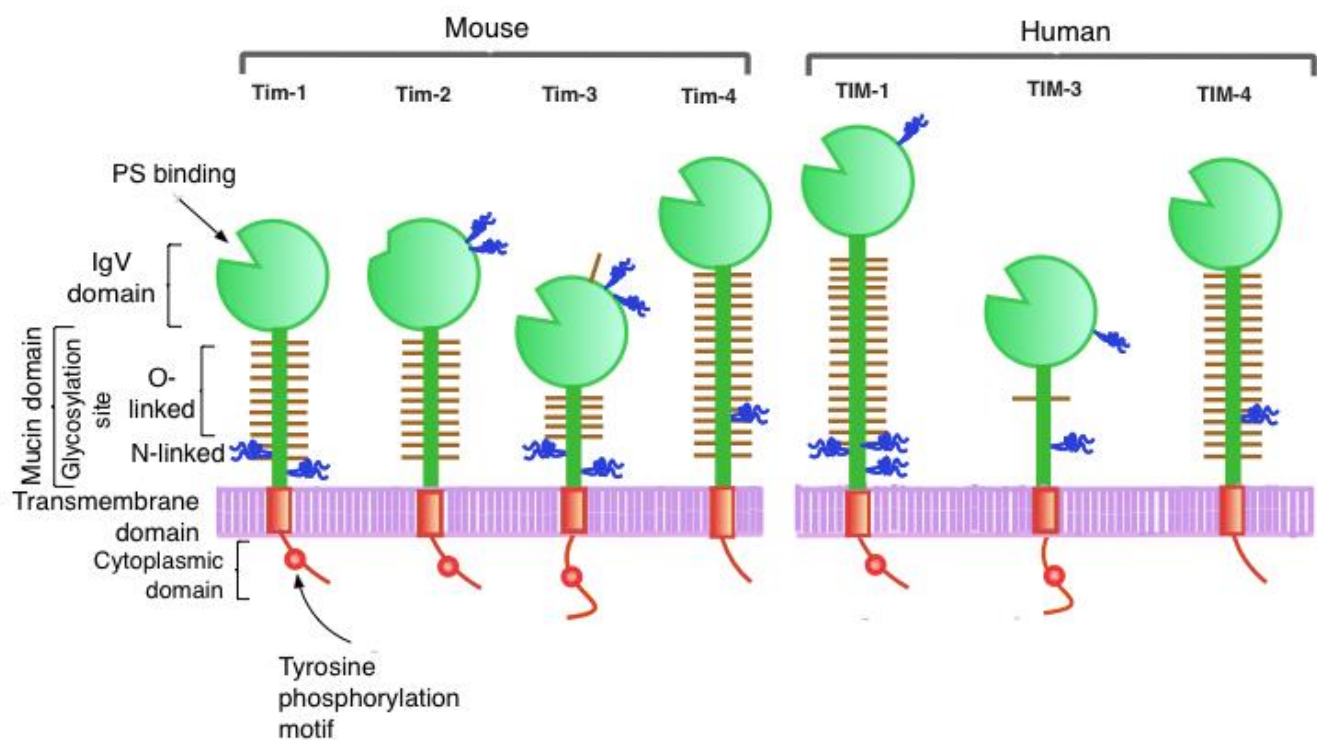


Figure 2-2: The TIM family proteins. Graphical representation of murine and human TIM proteins. Glycosylation sites in the IgV domain are indicated in blue, based on structural studies. Approximate sites of N- and O-linked glycosylation sites on the mucin domain are represented using NetOglyc and NetNglyc prediction servers.

2.3 TIM-1 (HAVCR-1)

2.3.1 Tim-1 expression

Mouse *Tim1* encodes a 305 amino acid (aa) membrane protein that has 78% identity with rat KIM1 and 42% identity with human HAVcr-1. KIM-1, or kidney injury molecule, is upregulated on kidney tubular epithelial following injury (200). Tim-1 is expressed on activated and Th2-polarized T cells but not naïve or CD8 T cells (201). Angiari *et al* recently reported that high levels of Tim-1 expression could be found both on the surface and intracellularly of Th1 and Th17 cells (202). Tim-1 is also expressed by DCs (203), germinal and regulatory B cells (204, 205), natural killer T (NKT) cells ((206) and mast cells (96).

2.3.2 Tim-1 as HAV cellular receptor and link to atopy

Human TIM-1 was originally identified as a receptor for HAV in African green monkeys and humans (207, 208). Sequence homology between murine Tim-1 and human TIM-1 allowed mapping of HAV binding to the IgV domain of Tim-1 (193). Tim-1 is highly polymorphic in mice, monkeys, and humans with variants ranging from single nucleotide to insertion/deletion polymorphisms primarily in the signal and mucin-like domains (190). Of interest is a six amino-acid-insertion (ins) at residue 157 termed the 157insMTTTVP located in the mucin domain that is required for efficient HAV uncoating and entry (209). HAV infection is commonly associated with large family size, poor hygiene and/or attendance at day-care; there is an inverse correlation between HAV exposure and atopy (210). By studying 375 individuals who were tested for atopy and prior HAV infection, McIntire *et al* found that individuals who were HAV seropositive were

protected against atopy, but only if these individuals also harbored the 157insMTTTVP variant of TIM-1 (211). These findings suggest that exposure to HAV (or possibly another pathogen that is also on a decline due to modern sanitization and public health measures) may influence the development of atopy. Subsequent to the study of McIntire *et al*, Tim-1 polymorphisms have also been associated with asthma, atopic dermatitis, allergic rhinitis, autoimmune diseases, malaria, AIDS progression and protection against malaria (212).

These disease associations are particularly important since an immune function for Tim-1 was originally identified by studying congenic mouse models differing in distinct chromosomal regions, to screen for asthma susceptibility genes. BALB/c mice had heightened AHR and Th2-type cytokine production when compared to HBA mice with chromosomal regions derived from the more asthma-resistant DBA/2 mice. These differences were later attributed to variation in the Tim proteins and their effects on T cell functions (190). However, human genetic linkage cannot fully explain the immune response differences observed between BALB/c and HBA mice, as BALB/c Tim-1 contains the longer mucin domain, yet also has a more atopic phenotype. Nevertheless, these results suggest that Tim-1 variation can regulate the balance of Th1-Th2 responses and provide a potential molecular mechanism for the inverse relationship between infection and atopy.

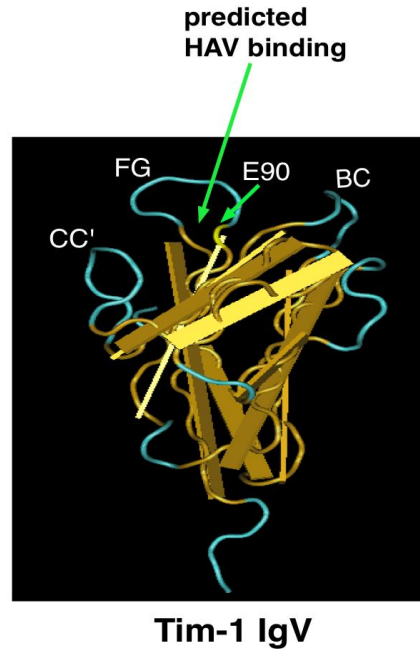


Figure 2-3: Tim-1 IgV domain and predicted HAV binding sites. Tim-1 IgV domain (PBD ID: 2OR8) was generated using Cn3D based on structural data in the public database. Predicted binding sites and the amino acid important for HAV interaction with Tim-1 are indicated.

2.3.3 Tim-1 as a phosphatidylserine (PS) receptor

PS is the most abundant phospholipid, with restricted distribution in the inner leaflet of the plasma membrane. Redistribution and exposure of PS to the cell surface is a cardinal feature of apoptosis and has been shown to facilitate clearance of apoptotic by phagocytes (213). TIM-1 is also known as kidney injury molecule (KIM-1), since it was found to be upregulated on tubular epithelial cells of post-ischemic kidney (200). Expression of Tim-1 on a renal cell carcinoma cell line mediated phagocytosis of apoptotic cells (214). Tim-1 ecto-domain can also be shed from injured kidney and is now used as a biomarker for post-ischemic kidney (200). Therefore, Tim-1 may function as a facilitator of cell death recognition and clearance in the injured kidney. As discussed below, Tim-

1 recognition of apoptotic cells is essential for activation of regulatory B cells in allograft tolerance and for natural killer T cells in non-allergic asthma (215, 216). Recently, TIM-1 has been shown to interact with PS to inhibit HIV release (217).

2.3.4 Tim-1 and other ligands

Tim-4, which is related to Tim-1, is also a ligand for Tim-1, and will be discussed in further detail below. IgA λ chain binds specifically to Tim-1 and can enhance HAV-Tim-1 interaction, without interfering with HAV infection, in African monkey kidney cells (218). As a result, IgA and HAV may have different binding sites on Tim-1 (218). Wilker *et al* proposed that Tim-1 and other Tim proteins can bind a large repertoire of ligands across species in a calcium-sensitive manner, with a requirement for intact O-linked and N-linked glycosylation (219). Specifically, mouse Tim-1 fusion protein could bind to mouse Tim-1, Tim-3 and Tim-4, as long as their mucin stalks were glycosylated (219). Finally, Tim-1 was recently reported to bind the selectin family member P-selectin (202). Tim-1 binding to P-selectin was shown to mediate Th1 and Th17 capture and rolling *in vitro* and *in vivo*, recruitment of T cells into inflamed skin in a contact hypersensitivity model and induction of EAE (202).

2.3.5 Tim-1 in T cells

Since the identification of Tim-1, many studies have focused on delineating its functions in T cells, using monoclonal antibodies and endogenous ligands (195, 201, 220, 221). Specifically, Tim-1 was shown to co-stimulate effector T cell proliferation, and has a preferential role in co-stimulation of Th2 cytokine production. Administration of the high affinity agonistic mAb 3B3 inhibited

induction of respiratory tolerance in an AHR model (201). Antibodies recognizing distinct epitopes of Tim-1 resulted in either enhanced or attenuated lung inflammation (220). Ligation of Tim-1 by a high affinity agonistic antibody was able to enhance T cell proliferation and cytokine production *in vitro* and *in vivo* (201, 222). Furthermore, anti-Tim-1 treatment was able to attenuate Th2-dependent airway inflammation in a mouse model of asthma by reducing inflammatory cell infiltration and Th2 cytokine production (223). This evidence suggests that Tim-1 plays a role in airway inflammation through its regulation of Th2 type responses that are central to development of AHR and the pathogenesis of asthma (206).

De Souza *et al* further showed that ectopic expression of Tim-1 in T cells stimulated *in vitro* under neutral conditions promoted generation of more IL-4 than INF- γ (222). Similarly, a co-stimulatory function of Tim-1 was also observed during interaction of Tim-1 with Tim-4, which primarily expressed on APCs (195, 224). Specifically, Tim4-Ig ligation of Tim-1 delivered a positive signal for CD3/CD28-mediated T cell proliferation. T cells from mice immunized with the encephalitogenic peptide PLP and treated with Tim4-Ig produce more IL-2 and IFN- γ than IL-4 and IL-10 (195). Collectively, these results provide supporting evidence that Tim-1 is a positive regulator of T cell response.

Tim-1 has also been implicated in transplant tolerance and the balance between effector and regulatory T cells. In transplant model studies, different Tim-1 antibodies demonstrated opposing effects on regulatory T cell differentiation and survival. For instance, the high affinity antibody 3B3 promoted graft rejection by reducing Foxp3 expression and Treg development while promoting Th17 responses (225). On the other hands, treatment with the low avidity antibody RMT1-10 was able to inhibit allograft rejection by inhibiting Th1 and Th17 responses while maintaining the Treg population (226).

2.3.6 Tim-1 in B cells

Studies of Tim-1 function using antibody treatment have been beneficial to detection of novel Tim-1 expressing cell types and the effects of modulation of its co-stimulatory activity. Similarly, using a mAb generated against murine Tim-1, Ma *et al* found that Tim-1 was highly expressed on splenic B cells after IgM and anti-CD40 stimulation (204). In addition, *in vitro* stimulation of activated B cells by Tim-1 Ab induced expression of the plasma cell marker syndecan-1. *In vivo* treatment with a Tim-1 Ab enhanced serum levels of antigen-specific antibodies, suggesting that Tim-1 signaling in B cells can regulate antibody responses (204). Another sub-population of splenic B cells, termed the regulatory B (Breg) cells (227), has been demonstrated to inhibit inflammation in autoimmune mice in an IL-10-dependent manner (228-230). Ding *et al* showed that Tim-1 expression could help define the IL-10-secreting Bregs and that Tim-1 ligation enhanced IL-4 and IL-10 expression in these B cells and prolonged allograft survival (231). The anti-Tim-1 Ab RMT1-10 was found to induce long-term allograft survival in mice when given in combination with rapamycin (226). It was recently found that combined treatment with RMT1-10 and anti-CD45RB could induce Tim-1 expression on splenic B cells and promote long term islet allograft survival (232). These results highlight the role of Tim-1 as a regulator of not only T cells but also B cell alloimmunity.

Since polymorphisms in the Tim-1 mucin domain can regulate Th2 responses in human and mice (190, 211), Xiao *et al* generated a Tim-1^{Δmucin} mouse model that expresses Tim-1 at normal levels but lacks the mucin (205). Tim-1^{Δmucin} mice appeared normal at <6 months of age but exhibited impaired IL-10 production by regulatory B cells at >10 months old. This was accompanied by hyper-activated T cell response and increased autoantibody production (205). These results suggest that the mucin domain is important for normal Breg function, absence of

which can lead to systemic autoimmunity. In addition, Tim-1^{Δmucin} exhibited accelerated allograft rejection, in part due to decreased Breg induction and IL-10 production in response to apoptotic cells (216). Overall, recent studies have demonstrated an important role for Tim-1 in the maintenance of tolerance specifically through promotion of regulatory B cell function.

2.3.7 Tim-1 and NKT cells

NKT cells are a distinct subset of T cells expressing the semi-variant TCR V α 14J α 18 that is restricted by MHC class I-like molecule CD1d (233). They express both the TCR/CD3 complex and the NK cell markers NK1.1 and Ly49 (233). Upon activation, NKT cells produce both Th1 and Th2 cytokines including IFN γ , IL-4, IL-10 and IL-13 (234, 235). As a result, NKT cells participate in a wide range of normal and pathological immune responses, such as tumor immunity, allergy, atherosclerosis and autoimmune diseases, yet the precise regulation of NKT cell cytokine production *in vivo* is not clear (206). Kim *et al* found constitutive expression of Tim-1 on NKT cells and, in the presence of TCR stimulation, ligation of Tim-1 either by antibodies or Tim-4 inhibited IFN- γ production and enhanced IL-4 production. This modulation of cytokine production was attributed to increased GATA-3 and reduced T-bet expression. Consequently, Tim-1 engagement aggravated bleomycin-induced pulmonary fibrosis, suggesting a regulatory role for Tim-1 in NKT-mediated disease (206).

NKT cells are abundant in the liver and have been associated with several forms of hepatitis (236). Kim *et al* reported TIM-1 expression on peripheral blood NKT cells and primary NKT cell lines, and that this expression was required for HAV-mediated activation of NKT cells, since only HAV-infected, but not uninfected, hepatoma cells were lysed (237). More importantly, examination of 30 Argentinean patients with HAV-induced acute liver failure revealed that severe

liver disease was associated with a 6-aa insertion (157insMTTTPV) in the *TIM1* gene, a polymorphism previously associated with protection against asthma and allergic diseases (211, 237). This is in part due to more efficient binding of HAV to cells expressing this long form of the mucin domain. These results provide a mechanistic explanation for the relationship among HAV infection, allergy and TIM-1, while highlighting the critical role of TIM-1 in immune regulation.

As a putative asthma susceptibility modifier, Tim-1 has a well-known role in asthma exacerbation, even though the mechanisms by which non-allergic environmental factors can trigger asthma is unclear. The capacity of Tim-1 to bind PS on apoptotic cells, and to regulate the severity of HAV infection and allergy, warranted an investigation into the role of Tim-1 in allergen- versus non-allergen induced asthma. Consequently, NKT cells were demonstrated to induce AHR in a respiratory syncytial virus (RSV)- and ozone-induced asthma, but not allergen-induced asthma, in a Tim-1-dependent manner (215). This study further showed that ozone and RSV exposure induced apoptosis of airway epithelial cells, which could activate NKT cells via Tim-1 signaling. On the other hand, two other studies reported either no difference or even increased AHR in allergen-induced lung inflammation in the absence of Tim-1 (238, 239). These results are particularly important because they suggest that Tim-1 may be involved in clearance of apoptotic cells, a critical function of NKT cells, caused by chronic lung inflammation, rather than production of inflammatory cytokines and recruitment of inflammatory cells.

2.3.8 Tim-1 in mast cells

Tim-1 is constitutively expressed on bone marrow-derived mast cells (BMMCs) and peritoneal mast cells (PMCs) (240). Cross-linking of Tim-1 by Tim-4 could enhance IgE-sensitized and antigen-stimulated (IgE/Ag) production of Th2 type cytokine production without affecting

degranulation (240). However, the mechanisms by which Tim-1 can modulate mast cell functional responses are currently unknown.

2.3.9 Tim-1 and dendritic cells (DCs)

Xiao *et al* showed that Tim-1 is expressed on all subsets of CD11c⁺ DCs but was higher on myeloid (CD11b⁺) and lower on plasmacytoid (B220⁺) DCs, and this expression was up-regulated by TLR activation with LPS (241). Cross-linking with the high avidity Tim-1 antibody 3B3 could enhance Th1 and Th17 cytokine production *in vitro* when DCs were present in the cultures and could also break tolerance in a genetically resistant mouse model of EAE (241). Tim-1 cross-linking on DCs may also alter the frequency of Treg, although this effect was rather modest. The preference toward Th1 and Th17 induction in this EAE model was attributed to 3B3-mediated activation of DCs, which represent the majority of Tim-1 expressing cells in the CNS at the peak of EAE severity (241).

2.3.10 Tim-1 signaling

Regarding signaling pathways coupled to Tim-1, de Souza *et al* showed that tyrosine 276 in the cytoplasmic tail of Tim-1 could be phosphorylated in an Lck-dependent manner (242). This allowed for recruitment of the p85 α and β subunits of PI3K, leading to activation of the downstream kinase Akt and subsequent activation of the transcription factors NFAT and AP-1 (242). Administration of the agonistic Tim-1 antibody 3B3 induces expression of early activation markers CD69 and CD25 as well as IL-2 production (242). Other groups have demonstrated that ligation of Tim-1 by Tim-4 can activate the ERK/MAPK pathway and enhance T cell survival by

upregulating the anti-apoptotic protein Bcl-x_L (224). Additional studies revealed that Tim-1 could co-cap with CD3 on human T cells (243). Tim-1 ligation on T cells has also been reported to induce tyrosine phosphorylation of the linker for activation of T cells (LAT) and the TCR-proximal Syk family tyrosine kinase Zap-70 (224). Taken together, these findings suggest that Tim-1 may interact with proximal TCR signaling complexes.

2.4 TIM-3 (HAVCR2)

2.4.1 Tim-3 expression

Tim-3 is a 281-amino acid protein that was initially identified as Th1 specific marker (191). Surface Tim-3 is now known to be expressed by a wide range of innate and adaptive immune cell types, including Th17, regulatory T cells, CD8⁺, natural killer (NK) cells, macrophages, monocytes, DCs and mast cells (96, 224, 227, 240, 244-246). An alternatively spliced form of Tim-3 that lacks the mucin and transmembrane domains exists as a soluble protein and, similar to soluble Tim-1 and Tim-4, could have relevant biological roles (247, 248). Regulation of Tim-3 expression requires the transcription factor T-bet (249). In addition, the mitogen-activated protein kinase (MAPK) is also involved in Tim-3 transcription in human CD4⁺ T cells and the human mast cell line HMC-1 (250). Recently, IL-27 was shown to cooperate with IL-10 to activate interleukin 3 regulated nuclear factor (NFIL3), leading to chromatin remodeling of the Tim-3 locus and induction of Tim-3 expression in Th1 cells (251).

2.4.2 Tim-3 and Galectin-9

Galectins, also called S-type lectins, are a family of carbohydrate-binding proteins expressed on a broad range of cell types and have crucial functions in regulating immune cell homeostasis and inflammation (252). Galectin-9 contains two distinct carbohydrate recognition domains connected by a long flexible linker, and exhibits enhanced affinity for poly-N-acetyllactosamine containing structure (253). IFN- γ and IL-1 β can upregulate galectin-9 expression in various tissues (254-256). Localized to the cytoplasm, galectin-9 is secreted through an unclear mechanism and subsequently binds to glycoproteins on target cells to exert its function. Galectin-9 is highly expressed on mast cells, and also found on T cells, B cells, macrophages, endothelial cells and fibroblasts (252). In T cells, galectin-9 is predominantly expressed on naïve CD4⁺ T cells and CD4⁺CD25⁺ regulatory T cells. Upon activation, galectin-9 expression is down-regulated on effector T cells but maintained on Treg (257, 258). Galectin-9 can also induce cell death of thymocytes and peripheral CD4⁺ and CD8⁺ T cells through a calcium-calpain-caspase-1-dependent pathway (259, 260).

Zhu *et al* showed that galectin-9 could bind to the IgV domain of Tim-3 in a glycosylation-dependent manner, and this binding induced Th1 cell death through a mixture of apoptotic and necrotic events (261). It is worth noting that galectin-9 binding is promiscuous and thus is not exclusive to Tim-3. Indeed, galectin-9 can bind and induce cell death of both Tim-3-deficient and wild type Th1 T cells, suggesting that galectin-9 can mediate its signal through other receptors. In addition, Su *et al* showed that galectin-9 could either induce apoptosis or secretion of pro-inflammatory cytokines, both of which occurred without Tim-3 activity (262). Furthermore, galectin-9 has been documented to interact with CD44 and IgE (263, 264).

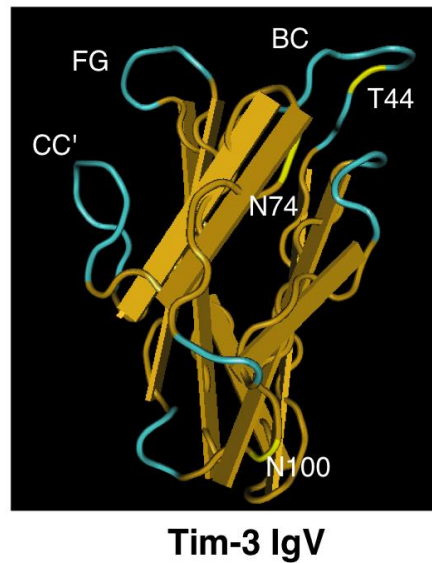


Figure 2-4: Tim-3 IgV domain and predicted galectin-9 binding site. Tim-3 IgV domain structure was rendered using Cn3D, based on public database entry (PDB ID: 2OYP). Potential glycosylation and galectin-9 binding sites labeled.

2.4.3 Tim-3 as a PS receptor

Tim-3 recognizes apoptotic cells by binding to PS via the FG loop of its IgV domain in a galectin-9-independent binding site (194, 265). Unlike Tim-4 which is expressed by peritoneal resident Mac1^+ (PRM) cells (266), Tim-3 is expressed by peritoneal exudate macrophages (26), suggesting different types of macrophages use different Tim molecules to mediate phagocytosis. Treatment with anti-Tim-3 Ab inhibited phagocytosis of apoptotic cells by peritoneal macrophages both *in vitro* and *in vivo*, leading to enhanced autoantibody production (265).

Tim-3 is expressed at high levels on CD8⁺, but not CD8⁻, splenic DCs. Antibody to Tim-3 inhibited by approximately 50% both recognition of apoptotic cells and cross-presentation by CD8⁺ DCs (265), suggesting that the Tim-3:PS interaction is important in phagocytosis. Since PS recognition by Tim-3 on lymphocytes does not induce phagocytosis, it is possible that this interaction triggers a pro-apoptotic signal on T cells similar to that of galectin-9 to Tim-3 on Th1 and Th17 (96, 261).

2.4.4 Tim-3 and HMGB1

HMGB1 (high mobility group protein B1) is a nuclear protein with essential roles in activating the innate immune responses mediated by the nucleic acid sensing systems (267, 268). In the nucleus, HMGB1 is a DNA-binding protein that acts to maintain genome stability and promote transcription, at the level of the chromatin (269). HMGB1 is translocated to the cytoplasm and released into the extracellular space, or can even be actively secreted, when cells are stressed, to regulate various aspects of immune responses, including inflammation, DC differentiation and autophagy (268, 269).

Chiba *et al* reported that Tim-3 could regulate nucleic acid-mediated innate immune responses by competing with nucleic acids for the DNA-binding A box of HMGB1 (270). As a result, Tim-3 was able to inhibit transport of nucleic acids to the endosome, an event normally mediated by HMGB1. Similar to PS, HMGB1 was shown to bind through the metal-ion-dependent ligand-binding site (MILIBS) in the FG loop (270). When tested in a tumor model, Tim-3 blockade was able to enhance anti-tumor activity when DCs were cultured with dying tumor cells exposed to cisplatin, a chemotherapy agent that induces “immunogenic” cell death (270). Thus, these

findings indicate that Tim-3 is a negative regulator of nucleic acid-mediated anti-tumor responses by circumventing HMGB1-nucleic acid transport pathways.

2.4.5 Tim-3 and CEACAM1

Recently, the carcinoembryonic antigen-related cell-adhesion molecule (CEACAM)-1 has been proposed as a ligand of Tim-3 (271). CEACAMs are expressed by epithelial, endothelial, lymphoid, and myeloid cells, and generally mediate intercellular adhesion through homophilic and/or heterophilic interactions (272). As a result, CEACAMs have well-established roles in tumor development, vascular neogenesis and apoptosis (272). CEACAM1 was detected at a low level on mouse T cells in the absence of inflammation as well as on resting human peripheral blood CD4⁺ T cells, and was rapidly upregulated on all T cells following stimulation with IL-2, IL-7, IL-15 or anti-CD3 antibody (272). It has also been shown that crosslinking of CEACAM1, by CEACAM1-specific antibodies, homophilic trans-ligation by soluble CEACAM1-Fc or with CEACAM1-transfected cells could inhibit T cell proliferation, cytokine production, and/or cytotoxicity (273-275). The CEACAM1 protein consists of an amino-terminal domain, a membrane distal IgV-like domain, followed by up to three membrane-proximal immunoglobulin-constant-region-type-2-like (IgC2-like) domains. The extracellular domains of CEACAM1 are heavily glycosylated. Homophilic intercellular binding is facilitated by a non-glycosylated β -sheet (276, 277). Huang *et al* demonstrated that CEACAM1 could mediate surface expression of Tim-3 by potentially interacting with Tim-3 in *cis* and *trans* through carbohydrate interaction between their extracellular domains (271). Furthermore, Tim-3 and CEACAM1 were co-expressed in a mouse model of OVA-induced tolerance, while CEACAM1-deficient CD4⁺ T cells had reduced surface expression

of Tim-3 (271). In a colorectal cancer model, a significant fraction of CD8⁺ tumor-infiltrating lymphocytes were triple positive for PD-1, Tim-3 and CEACAM1 and were characterized by extremely low levels of IL-2 and TNF- α . Finally, co-treatment with anti-CEACAM1 and anti-Tim-3 antibodies delayed tumor growth, with a greater level of protection than PD-1 and Tim-3 co-blockade (271). Overall, these results identify another mode of negative regulation by Tim-3/Tim-3L in immune regulation.

2.4.6 Tim-3 as a negative regulator of T cell responses

Initial studies using Tim-3 antibodies suggested that Tim-3 is a co-inhibitory molecule. For instance, administration of the anti-Tim-3 Ab (8B.2C12) during EAE induction exacerbated disease symptoms and increased the number of CD11b⁺ cells (191). Alternatively, treatment with a Tim3-Ig fusion protein resulted in hyper-proliferation of Th1 cells and increased Th1 cytokine production, possibly due to blockade of Tim-3/Tim-3L interaction (257). In addition, Tim-3 blockade by Tim3-Ig fusion protein accelerated diabetes onset in nonobese diabetic (NOD) mice and abrogated tolerance induction by co-treatment of donor specific transfusion and anti-CD40L Ab, possibly due to interference with regulatory T cell activity (258). Together with findings that Tim-3-deficient mice could not be tolerized and that galectin-9 could bind to Tim-3 to induce Th1 cell apoptosis, modulation of Tim-3/Tim-3L interaction emerged as a promising strategy to dampen Th1 responses and promote immunological tolerance.

An early focus in deciphering Tim-3 functions was on its regulation of the autoimmune disease MS using the mouse model EAE. Tim-3 was expressed by Th1, but not Th2, T cells. During development of EAE in SJL mice immunized with the myelin proteolipid protein (PLP) peptide, Tim-3 was upregulated on CD4⁺ and CD8⁺ T cells, and to a lesser extent on CD11b⁺ cells.

MS pathogenesis is associated with dysregulation of Th1 responses and production of pro-inflammatory cytokines IFN- γ and TNF- α , and treatment with galectin-9 reduced the number of IFN γ -producing cells and disease severity (261).

In humans, T cell clones isolated from cerebrospinal fluid of MS patients secreted higher levels of IFN- γ than clones from control cerebrospinal fluid, yet expressed a lower level of TIM-3. Furthermore, reduction of Tim-3 expression by small interfering RNA or Tim-3 blocking antibodies increased the number of IFN γ -producing CD4⁺ T cells, thereby supporting a negative regulatory of human TIM-3 in MS (278). Using *ex vivo* CD4⁺ T cells from healthy subjects and patients with MS, it was shown that blocking Tim-3 activity during T cell stimulation enhanced IFN- γ production in healthy controls but not in MS patients, suggesting dysregulation of Tim-3 regulatory pathway in MS. As a part of the dysregulated phenotype, Tim-3 in MS patients also exhibited reduced baseline level and delayed kinetics upon T cell stimulation, which could be rescued upon treatment with glatiramer and IFN- β , therapies that are effective in a subset of MS patients (279). These studies have proposed a strategy for therapeutic restoration of Tim-3 signaling pathway in correcting immunoregulation associated with MS.

In recent years, much attention has been given to Tim-3 as a mediator of T cell dysfunction in chronic viral infection. Virus-specific T cells in chronic human immunodeficiency virus (HIV) and hepatitis C virus (HCV) progressively develops a series of deterioration of T cell responses: loss of proliferative potential, IL-2, cytotoxicity and subsequently IFN γ production, a phenomenon that is referred to as T cell exhaustion (280, 281). Initial studies suggested that sustained expression of several inhibitory molecules, including program death (PD)-1, upon T cell activation was responsible for T cell exhaustion. In fact, blocking the PD-1/PD-1 ligand interaction in lymphocytic choriomeningitis virus (LCMV) in mice, simian immune deficiency syndrome in

rhesus macaques, and HIV in humans, has been demonstrated to decrease viral load *in vivo*, and enhanced T cell survival and proliferation of antigen-specific CD8⁺ T cells (280-282). Tim-3 expression on CD8⁺ T cells was associated with progressive HIV infection and correlated with disease progression (283). Specifically, there were two separate populations of Tim-3^{hi} and Tim-3^{lo} CD8⁺ T cells with the Tim-3^{hi} cells bearing the most diminished proliferative capacity and IFN- γ production, which could be reversed by treatment with soluble Tim-3-Ig (283). Consistent with their functional defects, Tim-3^{hi} T cells had impaired Stat5, Erk1/2, and p38 signaling upon stimulation, but also had higher basal phosphorylation of these proteins, suggesting the exhausted phenotype in Tim-3^{hi} T may be due to chronic basal activation rather than T cell unresponsiveness (283). More importantly, the Tim-3⁺ population is distinct from the PD-1⁺ population, thus raising the possibility of blocking both Tim-3 and PD-1 for effective anti-viral immune responses. In chronic HCV infection, Tim-3 expression was increased on both CD4⁺ and CD8⁺ T cells, and marked the cells with lower IFN γ - and TNF α -producing capability. Similarly, Tim-3 mAb treatment could reverse defects in cytokine secretion (284).

To understand how Tim-3 expression is correlated with viral infection, Jin *et al* followed Tim-3 expression on CD8⁺ T cells in acute and chronic LCMV infection (2010). Tim-3 was transiently expressed and rapidly down-regulated by CD8⁺ T cells in acute infection, but was maintained throughout chronic infection. Furthermore, Tim-3 was mainly co-expressed with PD-1 (PD-1⁺Tim-3⁺) on virus-specific CD8⁺ T cells, which represented the most exhausted phenotype compared to PD-1⁺Tim-3⁻ population T (285). Specifically, the PD-1⁺Tim-3⁻ population had three-fold higher proliferative capacity and two-fold higher IFN- γ , TNF- α and IL-2 secretion compared to the PD-1⁺Tim-3⁺ population in response to viral peptide GP33 stimulation. Since the PD-1⁺Tim-3⁺ also secreted high amount of IL-10, it is possible that this IL-10 producing CD8⁺ population

may exert suppressive effects on further limiting CD8⁺ T cell responses. Finally, blockade of Tim-3 synergized with anti-PDL1 treatment to reduce viral burden and enhance effector T cell functions (285). These findings further support a role of Tim-3 in T cell exhaustion and targeting Tim-3 and PD-1 as a more comprehensive reversal of T cell dysregulation.

Subsequence studies went on to characterize the mechanisms of T cell dysregulation in chronic viral infection. Sakhdari *et al* showed that while Tim-3⁺ CD8 T cells in HIV infected patients expressed high levels of granule-associated perforin, these cells were not able to degranulate and properly kill HIV infected autologous CD4⁺ T cells (286). CD8⁺ T cell cytotoxicity and target lysis were enhanced to that of healthy controls when treated with anti-Tim-3 Ab. While the mechanism of negative regulation mediated by Tim-3 is not well-understood, evidence suggests that Tim-3 can be cleaved by the matrix metalloproteinase ADAM10, resulting in a soluble form of Tim-3 (287), and the level of soluble Tim-3 in human plasma correlates with HIV disease progression (288). As a result, soluble Tim-3, like Tim-1, may represent a new marker for HIV pathogenesis.

Recently, much attention has been given to Tim-3 as an immune checkpoint receptor in tumor-induced immune suppression as Tim-3 was found on the most suppressed and dysfunctional tumor infiltrating CD8⁺ T cell (TILs) population in solid and hematologic malignancy (289, 290). In both of these models, PD-1⁺Tim-3⁺ TILs had defects in cell cycle progression and IL-2, IFN- γ and TNF- α production. In the colon adenocarcinoma model, Sakuishi *et al* demonstrated that the highest frequency of IFN γ -producing cells belongs to the Tim-3⁺PD-1⁺ TILs, an indication that PD-1 can mark both exhausted and effector T cells. More importantly, splenic CD8⁺ T cells did not harbor co-expression of PD-1 and Tim-3, suggesting that environmental cues could drive expression of PD-1 and Tim-3 (289). In the disseminated acute myelogenous leukemia (AML)

model, Tim-3 and PD-1 co-expression was acquired at late phase of disease progression and exhibited an effector phenotype (CD62L⁺CD44^{hi}) (290). Similar to TILs in solid tumors, Tim-3⁺PD-1⁺ TILs in AML secreted the least amount of IL-2, IFN- γ and TNF- α (290). Overall, the dysfunctional phenotype of Tim3⁺CD8⁺ T cells observed in tumor models resemble the exhausted CD8⁺ T cells in chronic viral infection. As a result, blocking both Tim-3 and PD-1 was able to inhibit tumor growth and reverse T cell exhaustion (289, 290).

The clinical relevance of Tim-3 blockade in anti-tumor immunity was supported in findings that Tim-3 is also found on T cells in patients with cancer. In patients with advanced melanoma, 30% of CD8⁺ T cells specific for the antigen NY-ESO-1 upregulated Tim-3 together with PD-1 (291). Tim-3/Tim-3L blockade synergized with PD-1/PD-1L interference to increase the frequency of cytokine-producing (IFN γ , TNF- α , IL-2) NY-ESO-1-specific CD8⁺ T cells (291). Tim-3 is also found on T cells of other cancers. Tim-3⁺ cells account for 30% of CD8⁺ TILs in patients with follicular B cell non-Hodgkin lymphoma (292). Tim-3 is highly expressed on CD4⁺ and CD8⁺ T cells in the tumors of patients with non-small cell lung cancer (NSCLC) and the majority of those Tim-3⁺ cells also express PD-1 (293). In all of these cancer types, Tim-3 is co-expressed with PD-1 and its expression is associated with defects in proliferation and cytokine production. Furthermore, in NSCLC tumor-mediated suppression was correlated with an increased frequency of Tim-3⁺Foxp3⁺ regulatory T cells, a subset of regulatory T cells with enhanced immunosuppressive capacity that has been observed in transplantable and *de novo* tumors (289). Collectively these data provide evidence for Tim-3 as a negative regulator in immune suppression and strongly support the rationale for Tim-3 blockade in the treatment of various cancers and chronic viral infections.

2.4.7 Tim-3 as a positive regulator of T cell responses

Recent studies of infectious diseases revealed that Tim-3 can also play a positive role in T cell effector responses to certain infections. Jayaraman *et al* showed that Tim-3 interaction with its ligand galectin-9 expressed on macrophages could stimulate macrophage activation and induce caspase- and IL-1 β -dependent bactericidal activity in response to *Mycobacterium tuberculosis* (*Mtb*) (294). When examining human patients with active TB, Qiu *et al* found that Tim-3 expression was upregulated on both CD4⁺ and CD8⁺ T cells, which was associated with greater production of IFN γ , TNF- α , IL-2 and IL-22 than their Tim-3⁻ counterparts (295). Furthermore, antibody cross-linking of Tim-3 on these T cells upregulated IFN- γ production, suggesting that Tim-3 signaling might actually contribute to enhanced anti-TB responses.

Gorman *et al* recently showed that Tim-3 may also play a supportive role in CD8⁺ effector function in response *Listeria monocytogenes* infection (296). Thus, Tim-3 expression was transiently upregulated on CD8⁺ T cells after *Listeria* infection, and was required for acquisition of an effector T cell phenotype. Tim-3-deficient mice exhibited impaired primary and secondary responses to *Listeria* that correlated with decreased IFN- γ production and degranulation (296). When transferred into wild-type hosts, Tim-3-deficient CD8⁺ T cells displayed impaired expansion and produced less cytokines compared to wild type control T cells. Collectively, these reports illustrated that the Tim-3-mediated balance between positive and negative effects during an immune response *in vivo* is complex and may be dependent on the location, duration and sequence of ligands interactions to generate an enhanced or diminished T cell response. The discrepancy with earlier studies also highlighted the need to better understand molecular mechanisms of Tim-3 effects on T cell activation and effector function.

2.4.8 Tim-3 and macrophages, monocytes, and dendritic cells

In addition to its complex function on T cells, Tim-3 also appears to have opposing effects on innate immune cells. Tim-3 is constitutively expressed on splenic DCs and treatment with galectin-9 synergized with TLR signaling to induce TNF- α production, likely through induction of NF- κ B transcriptional activation (227). TIM-3 staining was evident in white - but not gray - matter parenchyma on microglia (which are monocyte-derived) in the central nervous system (CNS), and was higher on monocytes and microglia in MS lesions, compared to control tissue (227). Galectin-9 expression is also upregulated on astrocytes in MS lesions relative to normal human CNS tissue. These findings suggest that TIM-3-galectin-9 interaction may help regulate Th1 responses. However, in mice immunized for induction of EAE, CD11b⁺ monocytes infiltrating the CNS and resident microglia upregulated Tim-3 expression compared to peripheral CD11b⁺ macrophages. Tim-3 antibody treatment actually exacerbated EAE disease severity (227). Thus, these initial studies demonstrated that Tim-3 activation could promote inflammation on innate cells, and dampen Th1 effector function once an adaptive immune response has been generated.

Subsequently, Tim-3 has been found to negatively regulate TLR signaling in macrophages to control sepsis, since blocking Tim-3 activity or overexpression of Tim-3 in macrophages led to enhanced sepsis or suppressed macrophage responsiveness to TLR4 signaling, respectively (297). Recently, Tim-3 on tumor-infiltrating DCs was found to interfere with nucleic acid sensing by interacting with HMGB1 (270). TIM-3 is constitutively expressed on resting human peripheral blood CD14⁺ monocytes/macrophages and is down-regulated upon TLR stimulation to allow for both IL-10 and IL-12 induction (298, 299). Thus, Tim-3 expression was inversely correlated with IL-12 secretion by human monocytes/macrophages in HCV-infected individuals while Tim-3 blockade or silencing Tim-3 expression could reverse IL-12 production, decrease PD-1 expression

and enhance Stat1 phosphorylation (298, 299). These findings suggest that Tim-3 plays both positive and negative roles in regulating inflammation in innate immune responses in a ligand- and cell type-dependent manner.

2.4.9 Tim-3 and natural killer (NK) cells

Tim-3 also has opposing effects on NK cell function, which may be disease-specific. Tim-3 was identified as an activating co-receptor for NK cells, as galectin-9 treatment significantly induced IFN- γ production and Tim-3 blockade diminished IFN- γ production by activated human NK cells (300). Tim-3 is highly expressed on mature CD56^{dim}CD16⁺ NK cells, and is further upregulated on CD56^{dim}CD16⁻ NK cells after stimulation with IL-15 or IL-12 and IL-18 in response to cytokine stimulation *in vitro* (301). While Tim-3 expressing NK cells are responsive to cytokine stimulation, cross-linking by Tim-3 antibodies can inhibit NK-mediated cytotoxicity (301). The presence of Tim-3⁺NK cells correlated with advancing stages of metastatic melanoma, and exhibited exhausted phenotypes characterized by decreased effector function, expression of activating receptors including NKG2D, and increased inhibitory receptors such as KIR3DL1. Similar to T cells, treatment with soluble antibodies to Tim-3 was able to reverse the exhausted NK cell phenotype and rescue cytokine production (302). Tim-3 was further described as an inhibitory modulator of NK cell function in LPS-induced endotoxic shock to restrain the excessive inflammation mediated by IFN γ -producing NK cells (303)). Finally, Wang *et al* showed that Tim-3 expression was upregulated on NK cells of patients with active TB, which correlated with impaired activation, degranulation and cytokine secretion (304). As NK cells activation utilizes a combination of inhibitory and activating co-receptors, it is important to examine how combined

Tim-3 engagement with specific NK cell co-receptors affect the functional outcome of NK cells in a particular disease setting.

2.4.10 Tim-3 and mast cells

Tim-3 is constitutively expressed on bone marrow-derived mast cells (BMMCs) and peritoneal mast cells (PMCs) and expression was increased six hours post IgE-sensitized antigen-mediated (IgE/Ag) activation (240). Cross-linking of Tim-3 with a polyclonal Ab was able to enhance antigen-induced IL-4, -5 and -13 production and to rescue IL-3-withdrawal-induced apoptosis in IgE/Ag-activated BMMCs, possibly by promoting IL-3 secretion from activated BMMCs. Nevertheless, Tim-3 cross-linking had no effect on mast cell degranulation (240). Transforming growth factor beta (TGF- β) has been shown to upregulate expression of Tim-3 in tumor infiltrating mast cells and a human mast cell line, through a mitogen-activated protein kinase Erk-kinase (MEK)-dependent pathway (305).

2.4.11 TIM-3 polymorphisms and association with allergic and autoimmune diseases

There are seven predicted amino acid differences between BALB/c and HBA Tim-3, and these polymorphisms cluster in the Ig domain (190). In fact, the allelic variants of BALB/c Tim-3 found in the BC loop of the IgV domain were responsible for the enhanced PS binding and phagocytosis of apoptotic cells compared to HBA Tim-3, likely by enhancing interaction with the surface of the lipid bilayer of the target cell (194). These initial results suggested that Tim-3 polymorphisms may have functional consequences in regulating Th1/Th2 responses.

Since the initial studies of DeKryuff and colleagues, many epidemiological studies have been conducted to determine if TIM-3 polymorphisms could affect human allergic and autoimmune diseases. In rheumatoid arthritis (RA), a Th1/Th17-mediated disease, there was an inverse correlation between TIM-3 expression and disease activity score. In addition, polymorphisms in the signal sequence and IgV domain were associated with RA in two Korean populations and a Chinese population (212). While the link was not as strong as in the case of TIM-1, TIM-3 polymorphisms have also been associated with susceptibility to Th2-mediated allergic disease. The -574T allele in the signal sequence was found only in asthma and allergic rhinitis patients, while the 4259T allele, which is close to the mucin region, was found more frequently in rhinitis patients in a Korean population. Meanwhile, another study of children from white or Hispanic parents found that two other polymorphisms, one in the signal sequence and one in the transmembrane domain, were associated with atopic dermatitis (212). Thus, many studies have documented the association of Tim-3 polymorphisms in atopy and autoimmunity in certain population, and Tim-3 may have an indirect effect on Th2 responses, due to its regulation of Th1 activity.

2.4.12 Tim-3 signaling

The cytoplasmic tail of Tim-3 contains six conserved tyrosine residues, some of which have been shown to couple to intracellular signaling pathways downstream of TCR/CD3, including data from our previous study (212, 306, 307). Specifically, Lck and the related Src family tyrosine kinase Fyn can interact with Tim-3, and Fyn can directly phosphorylate tyrosines within the Tim-3 cytoplasmic tail (308). Tim-3 phosphorylated in the region around tyrosines 256 and 263 can then recruit SH2-domain containing proteins including Fyn and the adaptor p85 of PI3K to mediate

downstream signaling (308). In addition, the Tec family kinase Itk has also been associated with Tim-3 phosphorylation at tyrosine 265 (tyrosine 256 in mouse) upon galectin-9 binding (307). Contrary to its apparent inhibitory role *in vivo*, ectopic expression of Tim-3 in human and murine T cell lines augmented CD3/CD28-stimulated NF- κ B and NFAT/AP1 activation in a manner that was dependent on the Tim-3 cytoplasmic tail, particularly residues Y256 and Y263 (308). Consistent with enhanced transcriptional activity, Tim-3 expression enhanced phosphorylation of PLC γ 1, ERK and ribosomal protein S6 downstream of the PI3K/Akt/mTOR pathway (308). In terms of T cell effector function, Tim-3 co-stimulation of TCR stimulation in primary murine T cells induced IFN γ production in a manner dependent on its cytoplasmic tail, and could be blocked by treatment of a Tim-3 mAb (308). Overall, these results indicated that, under acute stimulation, Tim-3 enhances signaling pathways leading to T cell activation, and can exert its effects without addition of exogenous ligands. Thus, these findings are in contrast to the role of Tim-3 as largely a negative regulator *in vivo* and will require further investigation to bridge these opposing findings.

Tim-3 has been shown to interact with the chaperone protein human leukocyte antigen B (HLA-B)-associated transcript 3 (Bat3) (309). Binding of Bat3 to Tim-3 cytoplasmic tail was able to prevent galectin9-mediated Th1 cell death and enhance IFN γ production. Since Bat3 can inhibit Tim-3 activity on Th1 cells, loss of Bat3 resulted in significant upregulation of surface Tim-3, reduced EAE severity and increased expression of exhaustion-associated markers (309). In terms of signaling mechanisms, Bat3 was reported to associate with the catalytically active form of Lck in a Tim-3-dependent manner (309). While Bat3 may be able to regulate Tim-3 expression and activity at a TCR-proximal level, it will be important to determine how TCR signaling can modulate the Tim3-Bat3 interaction.

A recent study on TIM-3 coordination in the formation of immunological synapse and proximal TCR signaling revealed that TIM-3 was recruited to the synapse and interacted with Lck in primary human CD8⁺ T cells (306). Specifically, Tim-3 recruitment was mediated by the phosphatases CD45 and CD148, and was enhanced by galectin-9 (306). As a result, Tim-3 may mediate its inhibitory function proximal to the TCR, by associating with phosphatases to disrupt synapse stability, since treatment with an antagonistic Tim-3 antibody resulted in more T cell-B cell conjugates with stable synapse (306).

2.5 TIM-4

Tim-4 was identified independently as SMUCKLER (spleen, mucin-containing, knockout of lymphotoxin), a gene downregulated in lymphotoxin (LT) α - and LT β -deficient mice (310). In the immune system, Tim-4 is expressed exclusively on antigen presenting cells, particularly macrophages and mature lymphoid DCs (195). Human TIM-4 is expressed by tinged-body macrophages in the germinal centers of tonsils and white pulp of spleen (214). Tim-4 is also a receptor for PS and has been shown to facilitate uptake of apoptotic thymocytes by mouse peritoneal macrophages via the conserved FG-CC' binding cleft in the IgV domain (214). Tim-4 was proposed to be a specific ligand of Tim-1, although Tim-1 and Tim-4 may bind to separate sites of PS-containing exosomes and thereby giving the appearance of an interaction (195, 266).

Unlike other Tim proteins, the cytoplasmic tail of Tim-4 lacks any defined signaling motifs and may therefore only function as a ligand, rather than a signaling receptor (190). Treatment with Tim4-Ig co-stimulated T cell proliferation *in vitro* and enhanced T cell hyperproliferation *in vivo*

(195). However, it should be noted that the effects of Tim-4 on T cell responses may be context-dependent, since Tim4-Ig was inhibitory to T cell proliferation at weak TCR stimulation, while acting as a co-stimulator under conditions of strong TCR stimulation (195). In terms of signal transduction, Tim4-Ig was shown to induce tyrosine phosphorylation of multiple substrates in T cells, as well as activation of Akt and ERK (224). Tim-4 can also play an active role in development of food allergy. Feng *et al* showed that exposure of bone marrow-derived DCs to cholera toxin and peanut extract upregulated Tim-4 expression and resulted in production of antigen-specific IgE and Th2 skewing in the intestine, when the pre-conditioned DCs were transferred to naïve mice (2008). Furthermore, pretreatment with anti-Tim-1 or anti-Tim-4 antibody was able to abrogate the Th2-skewed response. Similar effects were observed in an intestinal model of OVA and Staphylococcus enterotoxin B allergy induction (311).

2.6 TIM-2

The Tim-2 molecule has 85% nucleotide sequence identity with Tim-1, and is unique within the Tim family in that it does not have an ortholog in humans. In addition, Tim-2 does not possess the unique MILIBS pocket that confers specificity for phospholipid binding (193). Tim-2 is expressed mainly on APCs, including B cells and dendritic cells, as well as on non-immune cells of the bile duct epithelial in the liver, and hepatocytes (312). Tim-2 is not detected on naïve or activated T cells but is preferentially expressed on Th2-polarized T cells (313). Staining with a Tim2-Ig fusion protein identified Tim-2 ligands on activated DCs and macrophages (313). Indeed, Tim-2 was first identified to bind to the class IV semaphorin, Sema4A, a transmembrane protein expressed by DCs and B cells that can enhance T cell proliferation and cytokine production (198). Tim-2 was later

shown to facilitate uptake of H-ferritin, an iron-storing spherical protein complex that can regulate both non-immune and immune events (314). Crystal structure evidence revealed that Tim-2 can dimerize, which may prevent binding to other Tim proteins and facilitate binding of multivalent ligands (193).

Studies using Tim-2-Ig or Sema4A-Ig fusion proteins have identified Tim-2 as a Th2 regulatory molecule. *In vivo* administration of Tim-2-Ig resulted in splenic cell hyperproliferation, enhanced production of cytokines (IL-2, IL-4 and IL-10) and a delayed onset of clinical signs in an EAE model (313). In fact, Tim-2-Ig given just before disease onset could still reduce the symptoms of EAE. Based on the mechanisms proposed for Tim-3/Tim-3L, discussed above, it is possible that interaction of Tim-2 with its ligand(s) triggers inhibitory signals to downregulate Th2 responses. Thus, blocking this interaction could alter the balance of Th1/Th2 response even in a Th1-biased environment. Similarly, Sema4A-Fc could drive induction of both IFN γ - and IL-4-producing T cells, while anti-Sema4A antibody suppressed development of MOG-induced EAE when given at the time of MOG immunization (198). These protective effects were attributed to interaction with Tim-2, since Sema4A-deficient mice have impaired Th1 responses, and Tim-2-deficient T cells proliferated more than wild type T cells in response to antigen (315). The role of Tim-2 in negative regulation of Th2 responses was supported by the observation that Tim-2 knockout mice showed exacerbated lung inflammation (315). Moreover, biochemical analysis demonstrated that ectopic expression of Tim-2 could downregulate NFAT/AP1 transcriptional activity (316). Taken together, these findings demonstrate that Tim-2 expression on Th2 cells can modulate expansion and induction of Th2 responses.

3.0 STATEMENT OF THE PROBLEM

Ample evidence suggest that TIM family proteins have distinct expression patterns on a variety of cell types of both the innate and adaptive immune response. The task of deciphering how Tim-1 and Tim-3 function is complicated by the fact that they can each interact with multiple ligands or even with other TIM family members via their heavily glycosylated mucin regions (195, 219). As a result, the precise mechanisms of immunoregulation by Tim-1 and Tim-3 are not entirely clear and appear to be context- and ligand-dependent.

The *Tim* genetic locus was identified in a region of tightly linked genes that are implicated in asthma susceptibility (190). Since then, many studies have been conducted to determine how Tim-1 and Tim-3 modulate the balance between Th1/Th2 responses, especially since polymorphisms in *Tim-1*, and to a lesser extent *Tim-3*, are associated with differential susceptibility to atopy, allergy, viral infection and autoimmune diseases. In addition to T cells, Tim-1 and Tim-3 have also been found to regulate the function of innate cells, with critical roles in not only allergic inflammation and autoimmunity, but also in tumorigenesis and angiogenesis.

One of these innate cell types with high constitutive Tim-1 and Tim-3 surface expression is mast cells, which are first-line defenders against allergens and invading pathogens due to their proximity to the external environment. Antigen cross-linking of IgE bound to the high affinity IgE receptor FcεRI leads to the release of pre-formed mediators and *de novo* synthesis of pro-inflammatory and anti-inflammatory mediators and cytokines, which together serve to regulate hypersensitivity, autoimmunity, cardiovascular disease and tumor progression (317). In addition to their well-known pathologic roles in allergic responses, mast cells also contribute to defense against bacteria, helminths and tumors (318). While previous data suggest that Tim-1 and Tim-3

can function as positive regulators of mast cell activation, the molecular mechanisms behind their contribution to mast cell function are still unknown. Importantly, there was until now no genetic evidence addressing the function of Tim-1 and Tim-3 in these cells.

We hypothesized that Tim-1 and/or Tim-3 are costimulatory molecules of mast cell activation through FcεRI signaling to enhance mast cell mediator release. We plan to investigate the requirement of Tim-1 and Tim-3 in Ag-induced mast cell activation and how antibody modulation affects their activity on mast cells. Tim-1 and Tim-3 have been implicated in TCR proximal signaling (195, 219, 222, 242, 306-308, 319). Similarly, we intend to decipher the molecular mechanisms of Tim-1 and Tim-3 signaling at both the FcεRI proximal level and its downstream pathways. Specifically, we will determine whether mast cell Tim-1 and Tim-3 involve Src family tyrosine kinases and adaptor molecules similar to those in T cells as a mean of activation and signal transduction leading to enhanced cytokine production. Finally, we will examine the role of Tim-1 and Tim-3 *in vivo*, using a mast cell-dependent passive cutaneous anaphylaxis model. The results of these studies may identify one or more novel signaling pathways leading to mast cell activation by Tim-1 and Tim-3. Given the interest in immunotherapies targeting Tim-1 and Tim-3, and the multifunctional role of mast cells, characterization of Tim-1 and Tim-3 regulation of mast cell function could lead to development of more effective therapies to combat cancer, allergy and autoimmune disease.

4.0 TIM-3 CROSS-LINKING ENHANCES CYTOKINE PRODUCTION AND DEGRANULATION IN FcEPSILON-MEDIATED ACTIVATION OF BONE MARROW-DERIVED MAST CELLS

4.1 INTRODUCTION

Antibody(Ab)-mediated functional studies have led to the identification of Tim-3 as an essential regulator of immune functions. Treatment with Ab's to Tim-3 have been effective in blocking Tim-3 interaction with its ligands, leading to either exacerbated disease in T cell-mediated autoimmune settings, induction of peripheral tolerance, or reversal of T cell exhaustion in chronic viral infection and cancer (191, 257, 258, 261, 278, 284, 285, 289-291). On the other hands, antibody ligation of Tim-3 on innate cells could either promote monocyte activation in EAE, enhance DC-mediated anti-tumor efficacy, or inhibit NK cell activation (227, 301, 302, 320). Thus, antibodies to Tim-3 can be viewed as agonistic or antagonistic, depending on the cell types and ligands of interest.

Tim-3 is expressed constitutively by mast cells and can be engaged with a polyclonal Ab (Tim3 pAb) to enhanced FcεRI-mediated cytokine production (96). Here we examined the ability of other anti-Tim3 antibodies to co-stimulate FcεRI-mediated mast cell activation. In addition, we determined whether the effects of Tim-3 antibodies on mast cells might be the result of crosslinking-induced co-stimulatory activity or, as postulated in most T cell studies, an effect of Tim-3 “blockade.” Our data show that, upon antigen (Ag) ligation, treatment with Tim-3 antibodies enhances both mast cell degranulation and cytokine production. In addition, through analyzing mRNA expression data available from public database, we have compiled a list of genes

in mast cells that can be modulated by Tim-3 pAb cross-linking alone or in conjunction with Fc ϵ RI activation.

4.2 MATERIALS AND METHODS

4.2.1 Antibodies and reagents

Monoclonal anti-dinitrophenyl (DNP) antibody, IgE isotype, clone SPE-7, DNP₃₂ –HSA, 4-Nitrophenyl N-acetyl- β -D-glucosaminide (pNAG) were from Sigma-Aldrich (St. Louis, MO). Purified polyclonal antibody (Tim-3 pAb), directly fluorescent conjugated antibody to murine Tim-3, and normal goat IgG control were from R&D Systems (Minneapolis, MN). Purified mouse IgG control and mouse Fc block (clone 2.4G2) were from BD Biosciences (San Jose, CA). Monoclonal antibodies to murine Tim-3 (5D12, 3E3, 1B6) were obtained from Vijay Kuchroo (Harvard Medical School). Ionomycin was purchased from Calbiochem/EMD Biosciences (San Diego, CA)

4.2.2 BMMC isolation and culture

Bone marrow cells from C57BL/6 wild-type (WT) and knockout (KO) mice were cultured in RPMI 1640 supplemented with 10% BGS, non-essential amino acid, 2-ME, HEPES, pen/strep-glutamine, and 20% IL-3-conditioned media for 4-6 weeks, after which >90% of viable cells are mature mast cells (c-kit⁺ Fc ϵ RI⁺ by flow cytometry).

4.2.3 BMMC stimulation and cytokine measurement

BMMCs were sensitized with 1 µg/ml IgE overnight in complete media without IL-3. Cells were then stimulated either with DNP₃₂-HSA or DNP₅-BSA in IL-3-free media for indicated times. Supernatants were assayed for murine IL-6 and TNF-α by ELISA (BioLegend) six or twenty-four hours post-stimulation.

4.2.4 Mouse cytokine array

BMMCs were sensitized with IgE and stimulated with 30 ng/ml DNP₃₂-HSA in conjunction with either 5 µg/ml isotype control or Tim3 pAb for six hours. Supernatant was collected and subjected to analysis of cytokine release using the Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D Systems).

4.2.5 Beta-hexosaminidase release and flow cytometry assays for mast cell degranulation

BMMCs (2.5×10^5 cells) were stimulated as described above in Tyrode's buffer (135 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1.8 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, 0.5 mg/ml BSA). Thirty minutes post-stimulation, supernatants (stimulated release) were collected and cells were lysed with 200 µl of 0.5% Triton-X100 in PBS for fifteen minutes on ice. 20 µl each of lysate (content) and stimulated release were mixed with 20 µl of 1 mM pNAG substrate for 1 hour at 37°C. Reaction was stopped by addition of 200 µl of carbonate buffer (0.1M , pH 9.0) and absorbance was read at 405 nm. Percentage of beta-hexosaminidase release was calculated using the following equation: % release was calculated as $(\text{release}^{\text{stimulated}} / \text{content}^{\text{total}}) \times 100$.

Measurement of degranulation by flow cytometry was conducted as previously described (321, 322). Briefly, BMMCs were loaded with 0.1 μ M of LysoTracker Deep Red (Invitrogen) for thirty minutes at 37°C and then sensitized with 1 μ g/ml IgE for one hour. Receptor cross-linking was induced by addition of DNP₃₂-HSA at indicated concentration or 2 μ M ionomycin as positive control for ninety minutes prior to Annexin V staining (BioLegend). %degranulation is determined as percentage of BMMCs that is AnnexinV⁺LysoTracker^{lo}.

4.2.6 Microarray analysis from data available on GEO public database

The GEO dataset series GSE 14438 was obtained from National Center for Biotechnology Information (NCBI). Briefly, mouse *in vitro*-differentiated mast cells (BMMCs) were stimulated with Tim-3 pAb, isotype control alone or co-stimulated with IgE/Ag for two and sixteen hours prior to analysis with The Agilent Whole Mouse Genome Microarray kit. The NextBio platform (Illumina, Santa Clara, CA) was used to compare gene expression profile from the indicated stimulation conditions. The top genes with at least two-fold induction as a result of Tim3 pAb addition compared to isotype control were listed.

4.2.7 Statistical analysis

All statistical analysis was performed using Prism software (GraphPad Software). Paired, two-tailed Student's t-test, one-way and two-way ANOVA with Bonferroni post hoc test were used for data analysis and determination of p values, as appropriate.

4.3 RESULTS

4.3.1 Tim-3 antibodies enhance IgE/Ag-mediated cytokine and chemokine production in BMMCs

Nakae *et al* tested several antibodies for potential binding to Tim-3 and functional effects on mast cell activation (240). We confirmed that Tim-3 pAb enhanced IL-6 secretion in IgE/Ag-stimulated bone marrow-derived mast cells (BMMCs) six and twenty-four hours post stimulation (Fig.4-1A). In testing other Tim-3 antibodies, we found that 5D12, an antibody that is thought to antagonize the inhibitory activity of Tim-3 in T cells (227, 308), was able to enhance mast cell IL-6 and TNF- α cytokine release in BMMCs, albeit not to the same extent as a polyclonal Tim-3 Ab (pAb) (Fig.4-1B). We also determined that the 5D12 antibody alone does not induce cytokine production by mast cells (Fig.4-1C). Another, less well-characterized, monoclonal antibody (3E3) also exhibited co-stimulatory activity when combined with IgE/Ag treatment (Fig.4-1D). Finally, the monoclonal 1B6 antibody was not able to induce a statistically significant increase in mast cell activation (Fig.4-1E). Fc γ receptors, including Fc γ RIIB, are expressed abundantly on mast cells and have been shown to inhibit the induction of cellular activation programs in an ITIM-dependent manner (323). Therefore, we compared IL-6 release by BMMCs as above, with or without addition of an Fc γ R-blocking mAb. While Fc γ R binding was not a factor at the concentrations of Tim-3 pAb used throughout this study (5 μ g/ml), blocking Fc γ receptor binding to the Tim-3 mAb 5D12 actually further enhanced the agonistic effect of this antibody (Fig.4-2). Thus, the ability of Tim-3 antibodies to enhance mast cell activation was not due to Fc γ R binding.

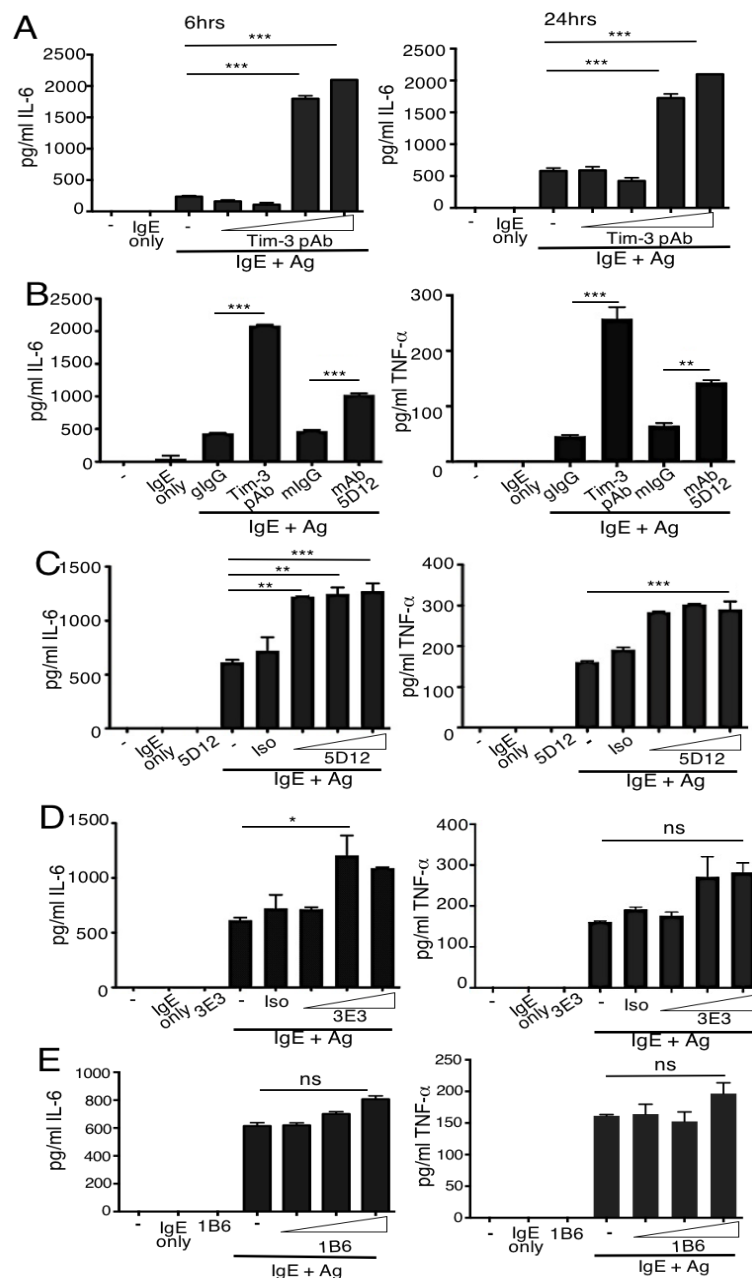


Figure 4-1: Tim-3 antibodies enhance IgE/Ag-mediated IL-6 and TNF- α production in BMMCs. BMMCs were sensitized with IgE overnight, then stimulated with either antigen alone, antigen with Tim-3 pAb, or isotype control for six and twenty-four hours (A). BMMCs were stimulated as indicated with antigen, antigen with Tim-3 pAb, mAb's 5D12, 3E3, 1B6, or the respective isotype controls, for six hours (B). Culture supernatants were collected and analyzed for IL-6 (left panels) or TNF- α (right panels) by ELISA. Results shown are representative of three independent experiments performed in duplicates. *p < 0.05, **p < 0.005, ***p < 0.0005.

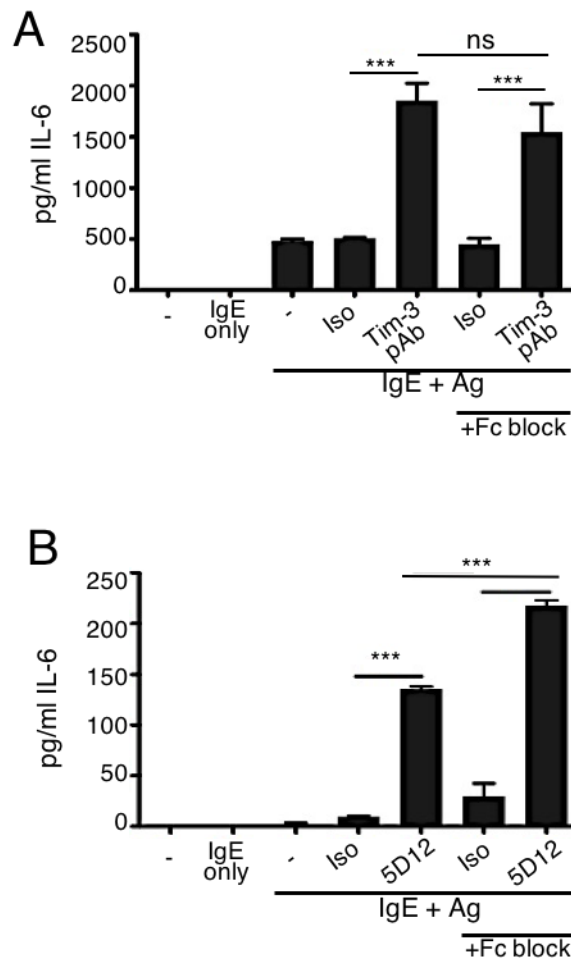


Figure 4-2: Tim-3 antibodies modulate mast cell activation independently of FcεRI signaling. BMMCs were sensitized and stimulated for six hours with antigen, in the presence of isotype control, Tim-3 pAb (A) or 5D12 (B), with or without Fc block (2.4G2). Supernatants were harvested and analyzed by ELISA for IL-6. Results shown are based on duplicate samples and are representative of two independent experiments. *** $p < 0.0005$.

4.3.2 Tim-3 antibody cross-linking exerts a modest but significant effect on IgE/Ag-mediated degranulation in BMMCs

As demonstrated before (240), Tim-3 antibodies did not affect Ag-induced mast cell degranulation as read-out by beta-hexosaminidase release thirty minutes after stimulation (Fig. 4-3A) . However, we were able to detect a small, but significant, increase in the percentage of mast cells degranulating at ninety minutes after stimulation, using a flow cytometry-based assay (Fig. 4-3B). In addition to yielding a higher signal-to-noise ratio that allowed for more accurate assessment of IgE/Ag-induced degranulation, this assay revealed that engagement of Tim-3 may need to be maintained for an extended period of time to mediate this particular co-stimulatory activity.

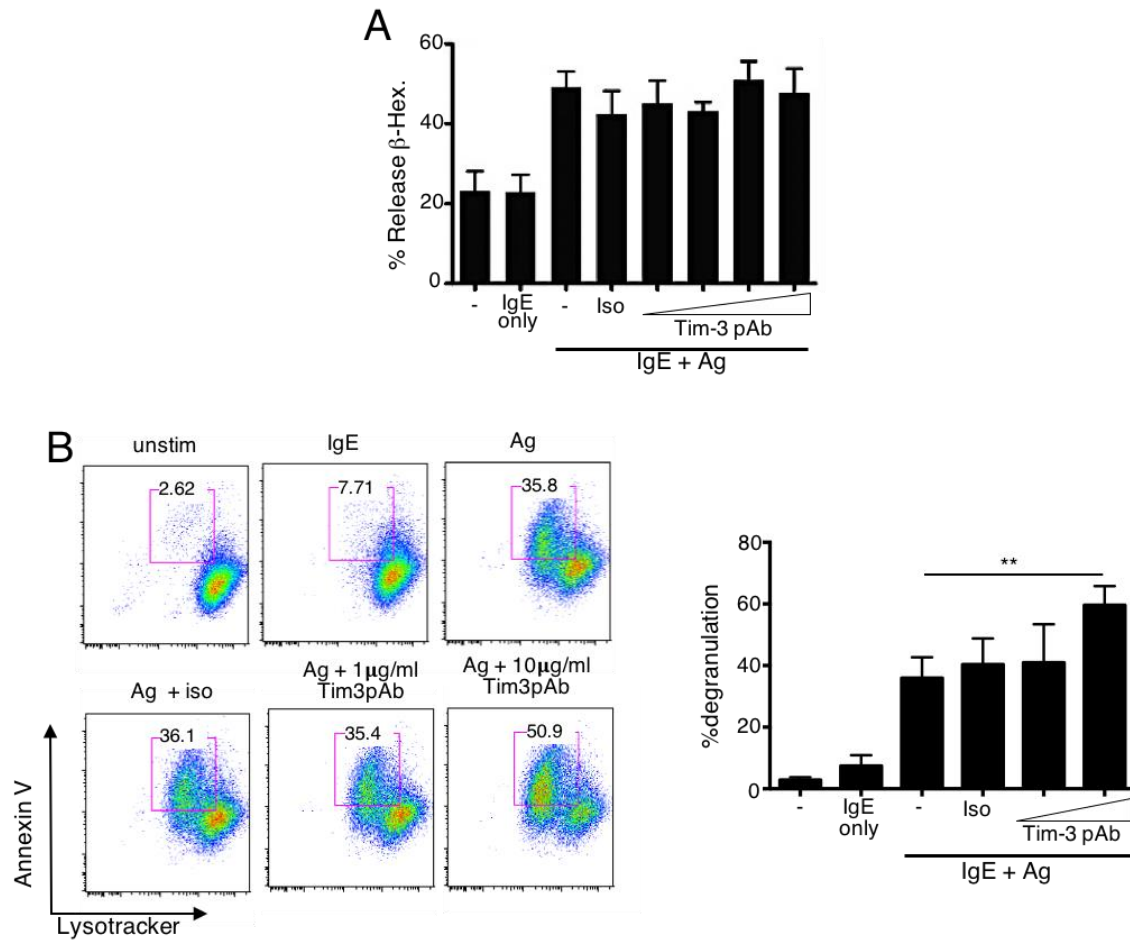


Figure 4-3: Tim-3 pAb enhances IgE/Ag-mediated degranulation in mast cells. BMMCs were sensitized overnight with IgE, stimulated with DNP₃₂-HSA alone or together with isotype control, or Tim-3 pAb, for thirty minutes. Degranulation was assessed by measurement of beta-hexosaminidase release (A); alternatively, cells were labeled with Lysotracker Deep Red, sensitized with IgE for one hour, and stimulated with antigen in the presence of isotype control or indicated amount of Tim-3 pAb for ninety minutes prior to Annexin V staining and flow cytometry analysis (B). Results shown are average of three independent experiments performed in triplicates (A) and duplicates (B). **p < 0.005.

4.3.3 Tim3 pAb co-stimulates protein and gene expression of various mast cell mediators upon FcεRI cross-linking

Mast cells can secrete a wide range of pre-formed and *de novo* synthesized mediators upon activation through its antigen receptor (32). To determine if the increase in cytokine production is specific IL-6 and TNF- α , we examined other mast cell products that may be affected by Tim3 pAb using a mouse cytokine array. Antigen stimulation alone induced production of well-known mast cell cytokines and chemokines including IL-6, IL-13, TNF- α , CCL2, and CCL3 (Fig.4-4A). While we did not detect expression or enhancement of IL-6 and TNF α , addition of Tim3 pAb induced production of a slightly different mediator profile including IL-7, IL-1 β , IL12p70, CCL4, and CCL12 (Fig.4-4B). In addition, we found a microarray study conducted to determine the genes affected by Tim-3 ligation alone or in co-stimulation with Ag (324). Thus, using the NextBio gene analysis platform, we observed that Tim-3 pAb alone induced a distinct gene expression profile compared to isotype control alone or IgE/Ag and Tim3 pAb co-treatment (Table1-4). Notably, the olfactory receptors, a family of G-protein-coupled receptors primarily expressed on the cell membrane of olfactory sensory neurons of the nasal epithelium for detection of odorant molecules (325), were robustly induced by Tim3 pAb alone (Table 1, 3). As the role of olfactory receptors on non-sensory cells are unknown, it remains to be seen whether olfactory receptor expressions on mast cells are functionally competent.

On the other hand, IgE/Ag and Tim3 pAb co-stimulation in just two hours enhanced expression of well-known mast cell mediator genes coding for IL-6, TNF, CCL2, and GM-CSF (Table 2). Of note, mast cell-derived amphiregulin, an epidermal growth factor (EGF)-like growth factor, has been shown to be critical for suppressive function of regulatory T cells in anti-tumor

responses (326). Tim-3 pAb co-stimulation showed almost six-fold induction of amphiregulin compared to isotype treatment, suggesting that engagement of Tim-3 on mast cells may indirectly influence anti-tumor activity through effects on regulatory T cells (Table 2). Mast cell *IL-2* gene expression was also upregulated over six-fold two hours post Ag stimulation, and thus represents another potential mechanism of mast cell modulation of T cell responses. Specifically, IL-2 secretion by mast cells is required for maintaining a balance between activated and regulatory T cells in suppression of chronic allergic skin inflammation (67). These results suggested that Tim-3 engagement can modulate release of a plethora of mediators that contribute to the multifunctional role of mast cells in the immune system.

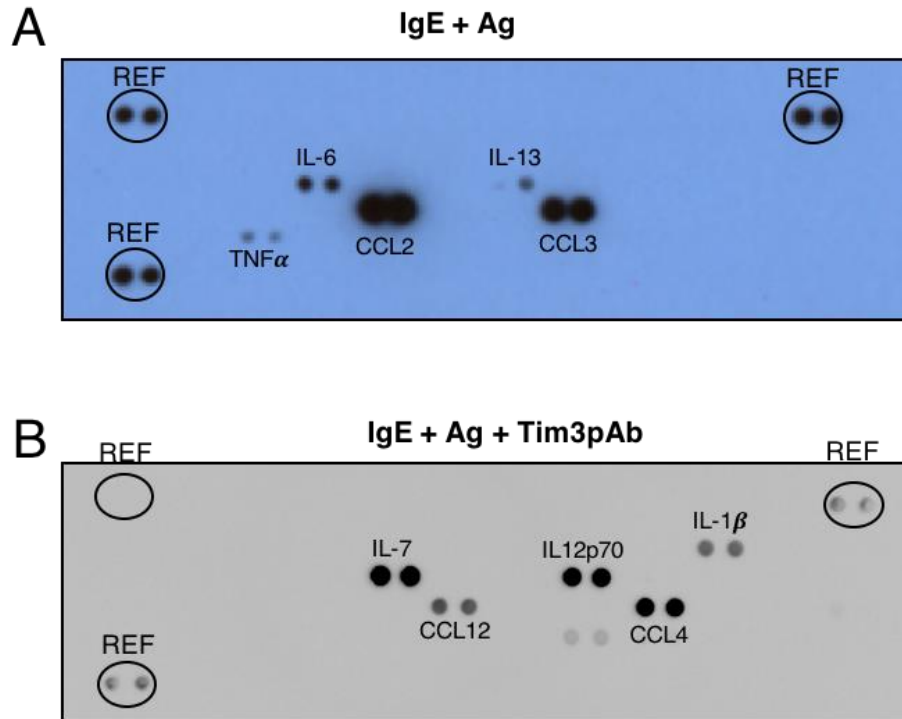


Figure 4-4: Tim3 pAb enhances a distinct pattern of mast cell cytokine production. BMMCs were sensitized with IgE and stimulated with 30 ng/ml DNP₃₂-HSA, with either 5 μ g/ml isotype or Tim3 pAb for six hours. Supernatant was collected and subjected to analysis of cytokine release using the Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D Systems).

Table 1: Gene expression induced by Tim-3 pAb stimulation alone for two hours

Gene name	Gene ID	Fold change	Notes
<i>Olfr1015</i>	NM_146571	6.45	Olfactory receptor 1015
<i>Gm8630</i>	AK083177	3.98	Predicted gene 8630
<i>6530403H02Rik</i>	AK032656	3.94	
<i>Ptprn2</i>	NM_011215	3.61	Protein tyrosine phosphatase
<i>Defb37</i>	NM_181683	3.46	Defensin beta 37
<i>Opn1mw</i>	NM_008106	2.94	Opsin 1
<i>Olfr112</i>	NM_001013575	2.68	
<i>Lphn3</i>	latrophilin 3	2.67	Latrophilin 3
<i>Wdr96</i>	AK014581	2.5	WD repeat domain 96
<i>Ccl3</i>	NM_011337	2.45	
<i>Ccl2</i>	NM_011333	2.31	
<i>Fasl</i>	NM_010177	1.94	

Table 2: Gene expression induced by IgE/Ag and Tim-3 pAb co-stimulation for two hours

Gene name	Gene ID	Fold Change	Notes
<i>Csf2</i>	NM_009969	10.6	GM-CSF
<i>Il6</i>	NM_031168	6.84	
<i>Il31</i>	AK005939	6.84	
<i>Ptgs2</i>	NM_011198	6.53	prostaglandin-endoperoxide synthase 2
<i>Ccl3</i>	NM_011337	6.46	
<i>Il2</i>	NM_008366	6.09	
<i>Areg</i>	NM_009704	5.75	Amphiregulin
<i>Map3k8</i>	NM_007746	5.61	Tpl-2
<i>Tnf</i>	NM_013693	5.5	TNF α
<i>Ccl2</i>	NM_011333	5.17	
<i>Il3</i>	NM_010556	5.16	
<i>Hbegf</i>	NM_010415	5.15	heparin-binding EGF-like growth factor
<i>Il11</i>	NM_008350	4.55	
<i>Il4</i>	NM_021283	4.46	
<i>Il13</i>	NM_008355	3.12	
<i>Gzmd</i>	NM_010372	2.98	Granzyme D
<i>Ifng</i>	NM_008337	2.8	

Table 3: Genes expression induced by Tim-3 pAb stimulation alone for sixteen hours

Gene name	Gene ID	Fold Change	Notes
<i>Lrp1b</i>	AK082259	6.13	low density lipoprotein-related protein 1B
<i>Olfir1015</i>	NM_146571	4.25	olfactory receptor 1015
<i>Defb37</i>	NM_181683	4.18	defensin beta 37
<i>Pigt</i>	NM_133779	3.99	phosphatidylinositol glycan anchor biosynthesis
<i>Cpm</i>	AK087332	3.41	carboxypeptidase M
<i>Efcab1</i>	AK030381	3.03	EF hand calcium binding domain 1
<i>Cntnap2</i>	AK043714	2.96	contactin associated protein-like 2
<i>Rapgef4</i>	NM_019688	2.62	GEF4
<i>Stk32a</i>	AK042850	2.53	serine/threonine kinase 32A
<i>Olfir112</i>	NM_001013575	2.44	olfactory receptor 112
<i>Grn</i>	NM_008175	2.39	Granulin
<i>Olfir1499</i>	NM_146796	2.3	olfactory receptor 1499
<i>Jakmip1</i>	NM_178394	2.24	janus kinase and microtubule interacting protein 1
<i>Cnr1</i>	NM_007726		cannabinoid receptor 1 (brain)
<i>Csf2rb</i>	AK154286	2.04	GM-CSF receptor beta

Table 4: Gene expression induced by IgE/Ag and Tim-3 pAb co-stimulation for sixteen hours

Gene name	Gene ID	Fold change	Notes
<i>1600029D21Rik</i>	NM_029639	7.22	RIKEN cDNA 1600029D21 gene
<i>Cd209a</i>	NM_133238	4.66	CD209a antigen
<i>Ccl1</i>	NM_011329	4.29	
<i>Gm8630</i>	AK083177	4.02	predicted gene 8630
<i>Gzmd</i>	NM_010372	3.95	granzyme D
<i>Ccl17</i>	NM_011332	3.74	
<i>Cntnap2</i>	AK043714	3.73	contactin associated protein-like 2
<i>Aqp1</i>	NM_007472	3.57	aquaporin 1
<i>Lrp4</i>	NM_172668	3.5	low density lipoprotein receptor-related protein 4
<i>Sucnr1</i>	NM_032400	3.49	succinate receptor 1
<i>Prss12</i>	NM_008939	3.38	Motopsin
<i>Opn1mw</i>	NM_008106	3.32	Opsin 1
<i>Lrp1b</i>	AK082259	3.21	low density lipoprotein-related protein 1B
<i>Ptprn2</i>	NM_011215	3.08	protein tyrosine phosphatase
<i>Olfr112</i>	NM_001013575	3.02	olfactory receptor 112
<i>Dnahc12</i>	XM_976110	2.95	Dynein
<i>Angpt1</i>	AK035599	2.94	Angiopoietin 1
<i>Prss37</i>	NM_026317	2.83	Serine protease 37
<i>Ramp2</i>	NM_019444	2.82	Calcitonin receptor activity modifying protein 2
<i>Hkdc1</i>	NM_145419	2.77	hexokinase domain containing 1
<i>Ccl24</i>	NM_019577	2.53	
<i>Entpd1</i>	NM_019577	2.52	ectonucleoside triphosphate diphosphohydrolase 1
<i>Il33</i>	NM_133775	2.29	
<i>NM_024435</i>	NM_024435	2.24	neurotensin

4.4 DISCUSSION

The main focus of this study was to determine how Tim-3 engagement by antibodies modulates mast cell activation and mediator release. This is a relevant issue, as various Tim-3 antibodies have been used in pre-clinical models, and are being considered for clinical use as immunotherapeutic agents (327, 328). Consistent with a previous report that Tim-3 pAb could co-stimulate FcεRI-

stimulated IL-4, IL-5 and IL-13 (240), we showed that this co-stimulation occurred in a dose-dependent fashion. We also identified other Tim-3 antibodies that could up-regulate Ag-stimulated cytokine production to varying degrees, notably the monoclonal antibody 5D12, which has often been used as an “antagonist” in studies of Tim-3 function in T cells (329-331).

Our decision to focus on Tim-3 engagement by antibodies was based in part on the non-specific binding of known Tim-3 ligands to other molecules on mast cells. Thus, phosphatidylserine (PS) can bind to several Tim family members besides Tim-3, including Tim-1 and Tim-4, through a conserved binding pocket in the Ig domain (332), in addition to binding to other cell surface receptors (333, 334). Galectin-9 has been shown to bind to Tim-3 and mediate down-regulation of Th1 immunity (261). However, galectin-9 is also an IgE-binding lectin and can downregulate allergic responses by disrupting IgE-Ag complex formation (264). Galectin-9 was also shown to bind to the receptor CD44, through which it can regulate inflammation at several levels (263, 335-337). Tim-3 expressed on tumor-infiltrating DCs interacts with the alarmin HMGB1, a DNA-binding protein associated with cellular injury, and interferes with an HMGB1-activated nucleic acid sensing system in the tumor microenvironment (270). However, HMGB1 can also bind to TLR4, which is expressed on mast cells, and the cellular receptor RAGE (338). Thus, engaging Tim-3 through antibody treatment offers a mechanism to more specifically modulate Tim-3 activity. Understanding how various antibodies can enhance or antagonize Tim-3 signaling will be important for the development of more effective antibody-mediated Tim-3 immunotherapies.

Through available gene expression data on public database, we were able to analyze on a broader scale how Tim-3 cross-linking by pAb impacted the expression profiles of FcεRI-regulated genes. Interestingly, Tim-3 pAb treatment alone induced a unique gene expression

pattern compared to IgE/Ag and Tim3 pAb co-stimulation. These results show that Tim-3 engagement alone can activate mast cells, at least at the transcriptional level, independently of FcεRI stimulation. While we did not detect any response in cells treated with Tim-3 pAb alone using cytokine production as a functional read-out, it remains to be seen whether the up-regulated gene expression profile represents a functional response and if Tim-3 pAb may be able to co-stimulate other receptors on mast cells.

5.0 TIM-3 KNOCKDOWN OR KNOCKOUT ATTENUATES ANTIGEN RECEPTOR-MEDIATED CYTOKINE PRODUCTION IN BMMCS

5.1 INTRODUCTION

While Tim-3 has been identified mostly as a negative regulator of T cell responses, recent studies hinted a possible positive role for Tim-3 in CD8⁺ T cell defense against *Listeria monocytogenes* infection (296). In contrary to observations in T cells, all of the Tim-3 antibodies tested in mast cells so far have a co-stimulatory function. Furthermore, when used in a mouse model of experimental allergic asthma, Tim-3-deficient mice were not impaired in development of lung inflammation (238). However, since the OVA-induced lung inflammation model used is primarily T cell-mediated and the role of mast cells was not examined, it remains to be seen whether Tim-3 deficiency will affect mast cell function *in vitro* and *in vivo*. Thus, we determined whether Tim-3 deficiency could impact the maturation and/or function of BMMCs, using Tim-3 knockout (KO) mice (296). Our findings demonstrated that acute knock-down or genetic deficiency of Tim-3 rendered mast cell less responsive to antigen cross-linking of FcεRI, resulting in decreased degranulation and cytokine production.

5.2 MATERIALS AND METHODS

5.2.1 Mice

Tim-3 KO mice were originally obtained from John Colgan (University of Iowa). The mouse strain had been back-crossed for at least 10 generations to C57BL/6 and was maintained in the University of Pittsburgh Animal Facility. Age-matched wild-type C57BL/6 mice (7-8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME)

5.2.2 BMMC isolation and culture

Bone marrow cells from Tim-3 WT and KO mice were cultured in RPMI 1640 supplemented with 10% BGS, non-essential amino acid, 2-ME, HEPES, pen/strep-glutamine, and 20% IL-3-conditioned media for 4-6 weeks, after which >90% of viable cells are mature mast cells (c-kit⁺ FcεRI⁺ by flow cytometry).

5.2.3 BMMC stimulation and cytokine measurement

BMMCs were sensitized with 1 µg/ml IgE overnight in complete media without IL-3. Cells were then stimulated either with DNP₃₂-HSA or DNP₅-BSA in IL-3-free media for indicated times. Supernatants were assayed for murine IL-6 and TNF-α by ELISA (Biolegend) six or twenty-four hours post-stimulation.

5.2.4 Flow cytometry assays for mast cell degranulation

Measurement of degranulation by flow cytometry was conducted as previously described (321, 322). Briefly, BMMCs were loaded with 0.1 μ M of LysoTracker Deep Red (Invitrogen) for thirty minutes at 37°C and then sensitized with 1 μ g/ml IgE for one hour. Receptor cross-linking was induced by addition of DNP₃₂-HSA at indicated concentration or 2 μ M ionomycin as positive control for ninety minutes prior to Annexin V staining (Biolegend). % degranulation is determined as percentage of BMMCs that is AnnexinV⁺LysoTracker^{lo}.

5.2.5 siRNA knock-down of Tim-3 in BMMC by nucleofection

BMMCs (3 x 10⁶ cells) were transfected with 100 pmol of non-specific or Tim-3 siRNA (Dharmacon) using the mouse macrophage nucleofector kit (Lonza), Y-001 program, and the Nucleofector II/2b device (Lonza). Transfected cells were collected after forty-eight hours and efficiency of knockdown was determined by Tim-3 staining, followed by flow cytometry.

5.2.6 Statistical analysis

All statistical analysis was performed using Prism software (GraphPad Software). Paired, two-tailed Student's t-test, one-way and two-way ANOVA with Bonferroni post hoc test were used for data analysis and determination of p values, as appropriate.

5.3 RESULTS

5.3.1 Tim-3 deficiency does not alter mast cell maturation but attenuates mast cell cytokine production and degranulation

Tim-3 deficient BMMCs exhibited normal development and maturation compared to WT controls, as judged by similar levels of c-kit and FcεRI expression at various times during their *in vitro* development (Fig. 5-1A). Strikingly however, Tim-3 KO BMMCs displayed a significant defect in cytokine production in response to FcεRI activation, using either low (DNP₅) or high (DNP₃₂) valency Ag (Fig. 5-1B). Of note, we observed significantly stronger stimulation with low valency Ag, likely due to the delayed engagement of negative feedback pathways that serve to down-regulate FcεRI signaling (109, 302, 339, 340). Thus, previous studies have noted the preferential engagement of positive or negative signaling pathways upon stimulation with low or high valency (or concentration) of Ag, respectively (109, 341). Importantly, the co-stimulatory effects of Tim-3 pAb or mAb 5D12 were severely impaired in Tim-3 KO BMMCs, which further reinforced the specificity of these antibodies for Tim-3 (Fig. 5-1C). In agreement with our findings (Fig. 4-3) that Tim-3 antibodies modestly enhance mast cell degranulation, Tim-3 deficiency impaired antigen-induced degranulation of BMMCs, using the flow cytometry-based assay (Fig. 5-1D). Thus, Tim-3 deficiency impaired the ability of BMMCs to generate robust responses to IgE receptor cross-linking, demonstrating that endogenous Tim-3 modulates the intensity of FcεRI signaling upon Ag challenge.

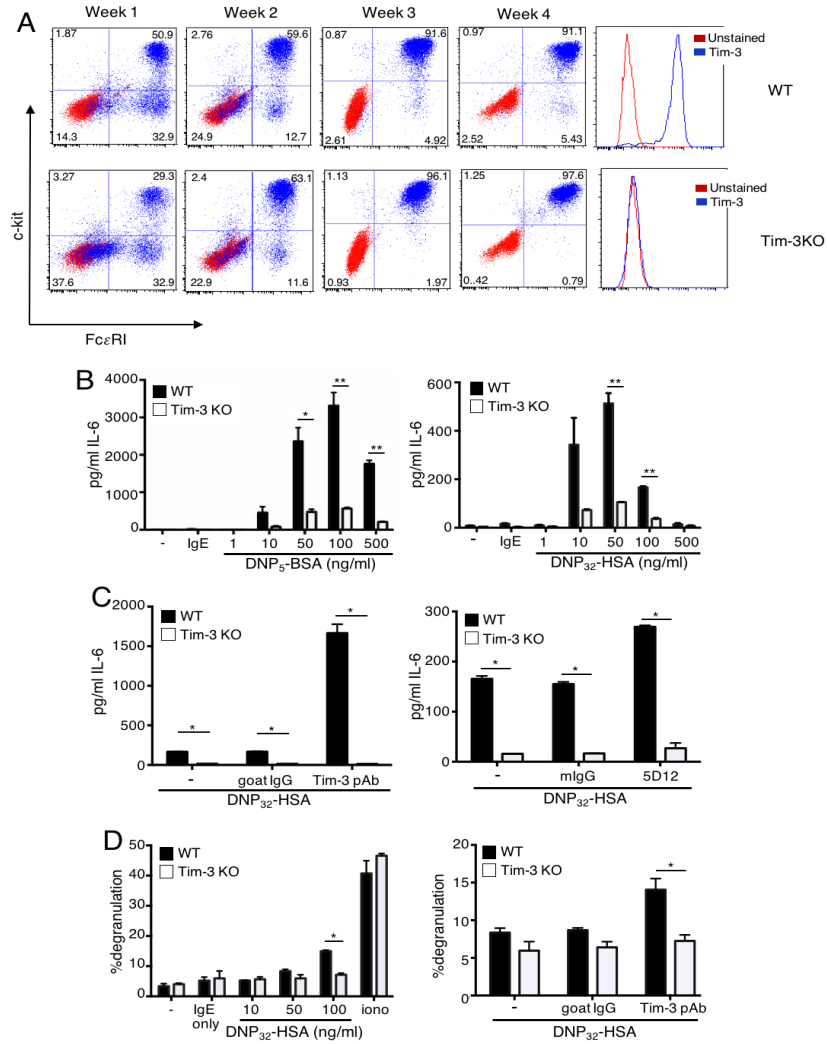


Figure 5-1: Tim-3 regulates cytokine production by BMMCs. (A) Maturity of Tim-3-WT and KO BMMCs as determined by FcεRI and c-kit staining. Tim-3 surface expression was compared between WT and Tim-3 KO BMMCs, which were sensitized with IgE and stimulated as indicated. Supernatants were collected after six hours and analyzed by ELISA for IL-6. (B) BMMCs were stimulated with low valency (DNP₅; left panel) or high valency (DNP₃₂; right panel) antigen. (C) BMMCs were stimulated with high DNP₃₂, plus isotype control, Tim-3 pAb (left panel) or Tim-3 mAb (right panel). Results are representative of three independent experiments performed for three batches of BMMCs. (D) BMMCs were stimulated with DNP₃₂ alone (left panel), or with isotype control or Tim-3 pAb (right panel), followed by quantitation of degranulation by flow cytometry. Results are representative of three independent experiments performed in duplicate. *p < 0.05, **p < 0.005.

5.3.2 Acute Tim-3 deficiency resulted in decreased cytokine production

Multiple *cis*- and *trans*-acting Tim-3 ligands have been described, and thus far include phosphatidylserine (PS), galectin-9, high mobility group protein B1 (HMGB1) and CEACAM1, none of which is specific to Tim-3 (296, 327, 330, 342). Furthermore, antibodies targeting Tim-3 have been shown to deliver either agonistic or antagonistic actions, as interpreted in the context of positive or negative effects that Tim-3 may exert on the specific cell type or disease model (191, 227, 291, 302, 343, 344). It was important to confirm that the effects of Tim-3 deficiency observed in the knockout mice were not due to secondary effects on mast cell development. Thus, we determined whether acute Tim-3 deficiency would have a direct impact on mast cell activation, by siRNA knockdown, while taking an agnostic view toward specific Tim-3 ligands. Consistent with a positive role of Tim-3, as described above, even incomplete knockdown of Tim-3 (~80% knockdown efficiency) resulted in a reduction of IL-6 production in Ag-stimulated BMMCs, a defect that was further emphasized when BMMCs were co-stimulated with Ag and Tim-3 pAb (Fig. 5-2A-B). Similar results were obtained across multiple batches of BMMCs, as well as when the anti-Tim3 mAb 5D12 was used, providing further evidence that Tim-3 functions as a positive regulator of FcεRI-induced cytokine production in mast cells (Fig. 5-2C). Furthermore, our experiments with Tim-3 KO BMMC confirmed that Tim-3 pAb and 5D12 are indeed specific to Tim-3.

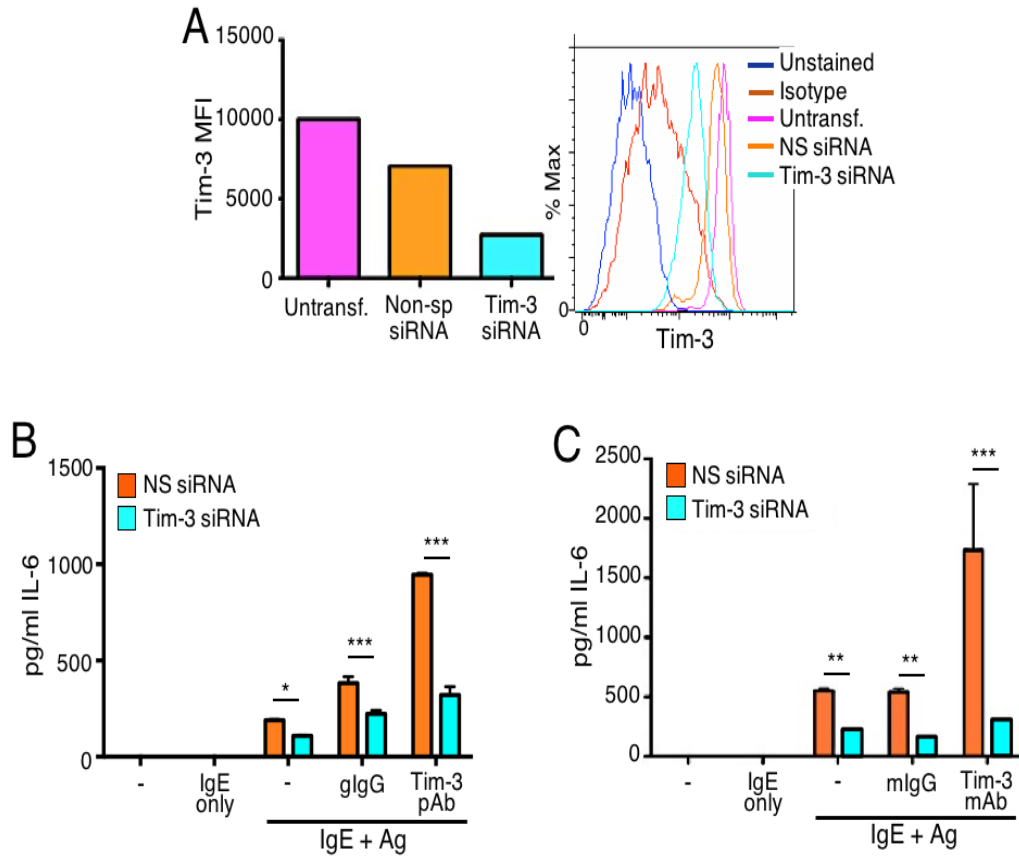


Figure 5-2: Tim-3 knockdown attenuates antigen-induced cytokine production by BMMC. BMMCs generated from C57BL/6 mice were transfected with control (non-sp) or Tim3-specific siRNA. (A) Forty-eight hours later, cells were analyzed by flow cytometry for Tim-3 expression. (B-C) BMMCs were sensitized overnight with IgE and stimulated with DNP₃₂-HSA for six hours. IL-6 secretion was assessed by ELISA, using either Tim-3 pAb (B) or 5D12 (C) for co-stimulation. Results are representative of three independent experiments, performed in duplicate. *p < 0.05, **p < 0.005, ***p < 0.0005.

5.4 DISCUSSION

We have demonstrated here that Tim-3 positively contributes to mast cells activation by IgE/Ag. To determine whether Tim-3 plays a positive or negative role in mast cell activation, we utilized both siRNA knockdown of endogenous Tim-3 in BMMCs, as well as a Tim-3 deficient mouse strain. Tim-3 knockdown and KO BMMCs exhibited defective degranulation and cytokine production at both high and low intensity IgE/Ag stimulation. It should be noted that the Tim-3-deficient mice used in this study was generated from the 129 strain embryonic stem cells and backcrossed to C57BL/6 mice for 10 generations. Because the *Tim* genes are tightly linked and are in close proximity with the pro-inflammatory IL-4 cytokine gene cluster, the carrying over of the 129 form of Tim-1 or neighboring genes should be taken into consideration as a potential confounding factor. Nevertheless, using siRNA-mediated knockdown, we showed that the impaired cytokine production observed was indeed due to reduced Tim-3 expression. Thus, Tim-3 amplifies the intensity and/or duration of signaling downstream of FcεRI, leading to cytokine production. Even though, Tim3 pAb and 5D12 could still enhance IL-6 release in Tim-3 siRNA-treated BMMCs since the knockdown was not complete, there was a complete absence of modulatory activity in the Tim-3 KO BMMCs, thereby further supporting the specific interaction between these antibodies and Tim-3.

6.0 TIM-3 REQUIRES TYROSINE PHOSPHORYLATION OF ITS CYTOPLASMIC TAIL TO AUGMENT MAST CELL ACTIVATION.

6.1 INTRODUCTION

The cytoplasmic tail of Tim-3 contains six conserved tyrosine residues, at least some of which have been shown to couple to the intracellular signaling network downstream of TCR/CD3, based on our previous study (308). Contrary to its apparent inhibitory role *in vivo*, ectopic expression of Tim-3 in human and murine T cell lines augmented CD3/CD28-stimulated NF- κ B and NFAT/AP1 activation in a manner that was dependent on the Tim-3 cytoplasmic tail, particularly residues Y256 and Y263 (308). Similarly, a conserved tyrosine on cytoplasmic tail of human TIM-3 was also phosphorylated upon galectin-9 ligation (306). Therefore, we asked whether over-expression of Tim-3 in a mast cell line could also augment transcriptional activation by antigen receptor signaling. Based on the increase in IL-6 and TNF- α and our findings in T cells, we examined transcriptional activity of the transcription factors NF- κ B, NFAT, and AP1. Our findings support the importance of tyrosine phosphorylation in Tim-3-mediated signaling as well as reveal a Syk-dependent regulation of Tim-3 activity downstream of IgE receptor cross-linking.

6.2 MATERIALS AND METHODS

6.2.1 Antibodies and reagents

Monoclonal anti-dinitrophenyl (DNP) antibody, IgE isotype, clone SPE-7, anti-Flag antibody M2, DNP₃₂-HSA, and anti-FLAG M2 antibody were from Sigma-Aldrich (St. Louis, MO). Phorbol myristate acetate (283), ionomycin, inhibitors to Src-family kinases (PP2), and Syk (BAY61-3606), were purchased from Calbiochem/EMD Biosciences (San Diego, CA). IL-6 luciferase reporter constructs (full length, mutants lacking binding sites for NF- κ B, AP1, C/EBP, NF- κ B/C/EBP) were obtained from Sarah Gaffen (University of Pittsburgh), and were originally obtained from Oliver Eickelberg (Helmholtz Zentrum Munchen). Phospho-Src (Y416) was purchased from Cell Signaling Technology (Danvers, MA). Total tyrosine phosphorylation antibody 4G10 was purchased from Millipore (Darmstadt, Germany).

6.2.2 Transcriptional luciferase assays

MC/9 mast cells (15×10^6 cells) were transfected with 15 μ g of NF- κ B-luc, NFAT/AP1-luc, NFAT-luc, AP-1-luc, IL-6-luc together with indicated amount of pCDEF3, FLAG-tagged Tim-3 full length, truncation 1 or 2 mutants (T1 or T2) by electroporation at 290 V, 950 μ F using a Gene Pulser II apparatus (Bio-Rad). Twenty-four hours later, transfected cells were collected and stimulated with 0.5 μ g/ml IgE and the indicated amount of DNP₃₂-HSA antigen for 6 hours. Luciferase assays were conducted as described previously.

6.2.3 Western blotting

MC/9 were sensitized with 0.5 µg/ml IgE for two hours at 37°C in Tyrode's buffer. Cells were then pre-treated with indicated concentration of Src or Syk inhibitors for thirty minutes prior to stimulation with DNP₃₂-HSA for ten minutes in Tyrode's buffer. Cells were lysed in 1% Nonidet-P-40 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) supplemented with AEBSF, aprotinin, leupeptin, pepstatin, sodium fluoride, sodium orthovanadate, and beta-glycerophosphate on ice for twenty minutes. Lysates were centrifuged at 4°C for fifteen minutes. For western blotting analysis, SDS-containing sample buffer was added to lysates prior to loading onto 10% SDS-PAGE gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using the PierceG2 fast blotter (Pierce), probed with appropriate antibodies, and imaged on a ProteinSimple Fluochem M cooled CCD imager (ProteinSimple).

6.2.4 Statistical analysis

All statistical analysis was performed using Prism software (GraphPad Software). Paired, two-tailed Student's t-test, one-way and two-way ANOVA with Bonferroni post hoc test were used for data analysis and determination of p values, as appropriate.

6.3 RESULTS

6.3.1 Transient expression of Tim-3 enhances IgE/Ag-mediated NF- κ B, NFAT/AP1, and IL-6 transcriptional activation

We expressed our previously described full-length (FL) murine Tim-3, a truncation (T1) lacking the three most distal C-terminal tyrosines, and a truncation (T2) lacking all but one membrane-proximal tyrosine, in the mouse mast cell line MC/9 (Fig. 6-1A). Similar to our findings in T cells, FL Tim-3 efficiently augmented NF- κ B, NFAT, AP1, as well as NFAT/AP1 composite reporter activity, compared to empty vector control, in IgE/Ag-stimulated mast cells (Fig. 6-1B-E). The cytoplasmic tail was important for Tim-3 signal transduction in mast cells, as all reporter co-stimulatory activity was abrogated when the T2 Tim-3 construct was expressed. While induction of NFAT/AP1 activity required mainly the more N-terminal tyrosines (Fig. 6-1C), NF- κ B transcriptional activity was sensitive to deletion of both N- and C-terminal tyrosines (Fig. 6-1B). Intriguingly, the T2 construct appeared to possess dominant negative activity in these assays. We did not observe further augmentation by Tim-3 upon cross-linking by Flag antibody, further supporting our previous findings that Tim-3 ectopic expression was sufficient to drive Ag-mediated transcriptional response.

Since Tim-3 cross-linking enhanced IL-6 secretion, and there are binding sites for NF- κ B, AP1 and C/EBP in the IL-6 promoter (306, 345-349), we examined the IL-6 promoter to determine if it was impacted by Tim-3 overexpression. As expected, FL Tim-3 augmented IL-6 promoter activity in a dose-dependent manner (Fig. 6-1F). Consistent with the requirement for AP-1 and NF- κ B in IL-6 reporter activation, Tim-3 could not augment IL-6 reporter activation in the absence

of NF- κ B or AP-1 (Fig. 6-1G). On the other hand, C/EBP was not involved in Tim-3-mediated IL-6 transcriptional activity (Fig. 6-1H). These results suggest that Tim-3 exerts its co-stimulatory effect on IL-6 production through transcriptional activation of NF- κ B and AP-1, which in turns drives IL-6 promoter activation and cytokine production.

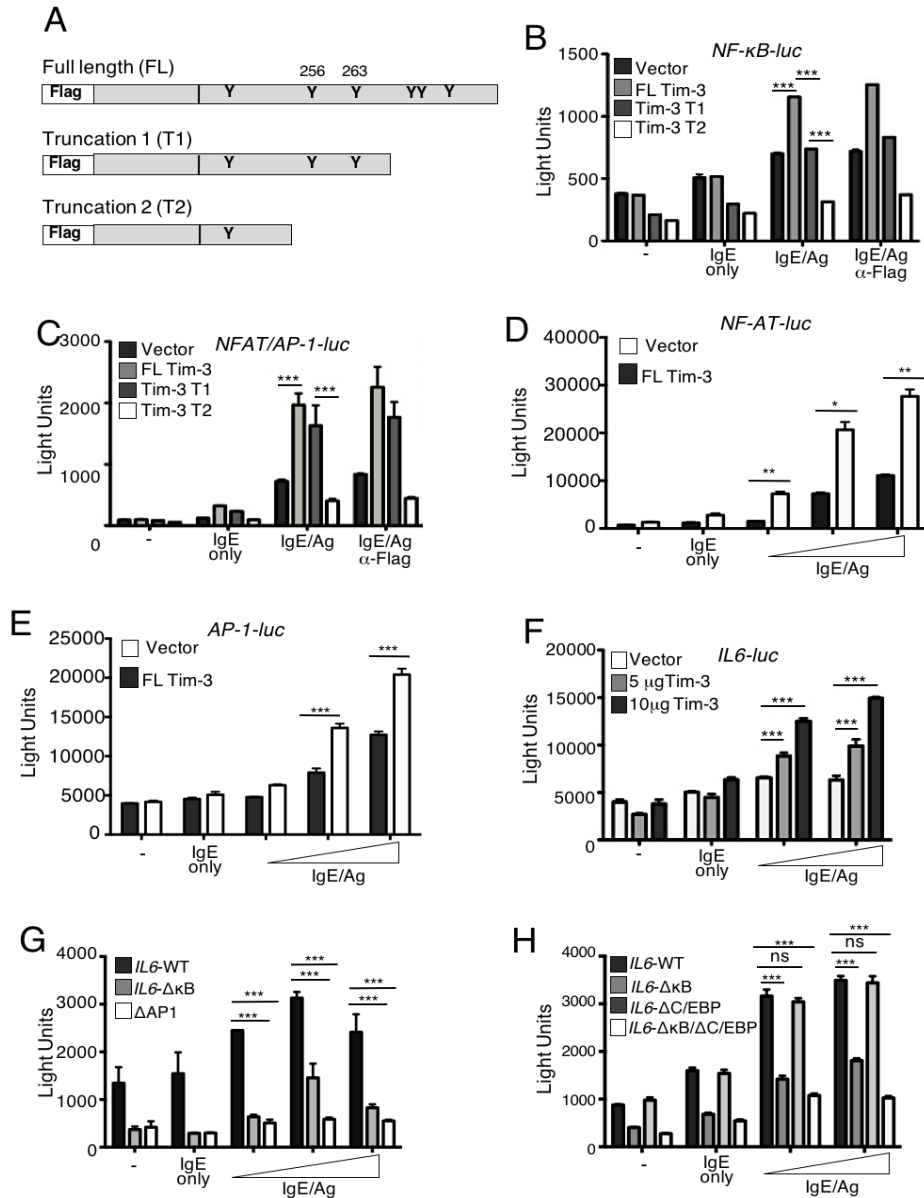


Figure 6-1: Tim-3 cytoplasmic tail tyrosines are required to augment NF- κ B, NF-AT, AP-1, and IL-6 transcriptional activation. MC/9 mouse mast cells were transfected with empty vector (pCDEF3) or one of the indicated Flag-tagged Tim-3 constructs (A). Transfected MC/9 cells were stimulated with IgE and 50 ng/ml or 100 ng/ml of DNP₃₂-HSA, with or without addition of anti-Flag antibody for six hours. (B-F) MC/9 mast cells were co-transfected with luciferase reporters for NF- κ B (B), NFAT/AP-1 (C), NF-AT (D), AP-1(E), or IL-6(F). (G-H) MC/9 mast cells were co-transfected with FL-Tim-3 and the indicated IL-6 reporter mutants. Results are representative of three independent experiments performed in triplicate. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

6.3.2 IgE/Ag-mediated IL-6 reporter activation depends on Syk but not Src family kinases, and is mediated by tyrosine phosphorylation of Tim-3 cytoplasmic tail

Next, we defined the role of known FcεRI signaling intermediates in Tim-3 co-stimulation of mast cells. Using IL-6 reporter activity as a read-out, we treated FL Tim-3-transfected MC/9 mast cells with the Src family tyrosine kinases (SFK) inhibitor PP2 or the Syk kinase inhibitor BAY61-3606, which demonstrated potent inhibition of Src phosphorylation and total tyrosine phosphorylation, respectively (Fig. 6-2A-B). IL-6 reporter activity was severely impaired in the presence of the Syk inhibitor, consistent with a strict requirement for Syk in FcεRI signaling (350, 351) and overexpression of Tim-3 did not rescue this inhibition (Fig.6-2C). By contrast, PP2 inhibited IL-6 reporter activity only at high concentrations (10 μM), where pSrc was severely diminished (Fig. 6-2B), while lower concentrations of PP2 actually enhanced IL-6 promoter responses (Fig. 6-2D). Tim-3 co-stimulatory activity was also similarly modulated by high vs. low concentrations of PP2 (Fig. 6-2D).

Several SFK family members are expressed in mast cells, including Lyn, Fyn and Hck, whose positive and/or negative regulatory function can be differentially modulated using either high or low dose PP2 (340). Thus, low dose PP2 appears to selectively block the negative regulatory functions of SFKs, while high-dose PP2 preferentially affects the positive function. Returning to the tyrosines within the Tim-3 cytoplasmic tail, we previously showed that phosphorylation of tyrosines 256 and 263 in the Tim-3 cytoplasmic tail is essential for its co-stimulatory activity in T cells (308). Consistent with this finding, a full length Tim-3 construct harboring tyrosine to phenylalanine mutation at these sites (Y256/263F) also abrogated Tim-3-augmented IL-6 reporter activation in mast cells. Phosphorylation of tyrosines 256 and 263 appears

to be the dominant mode of activation, as additional deletion of the three C-terminal tyrosines did not result in further reduction of IL-6 reporter activity (Fig. 6-2E).

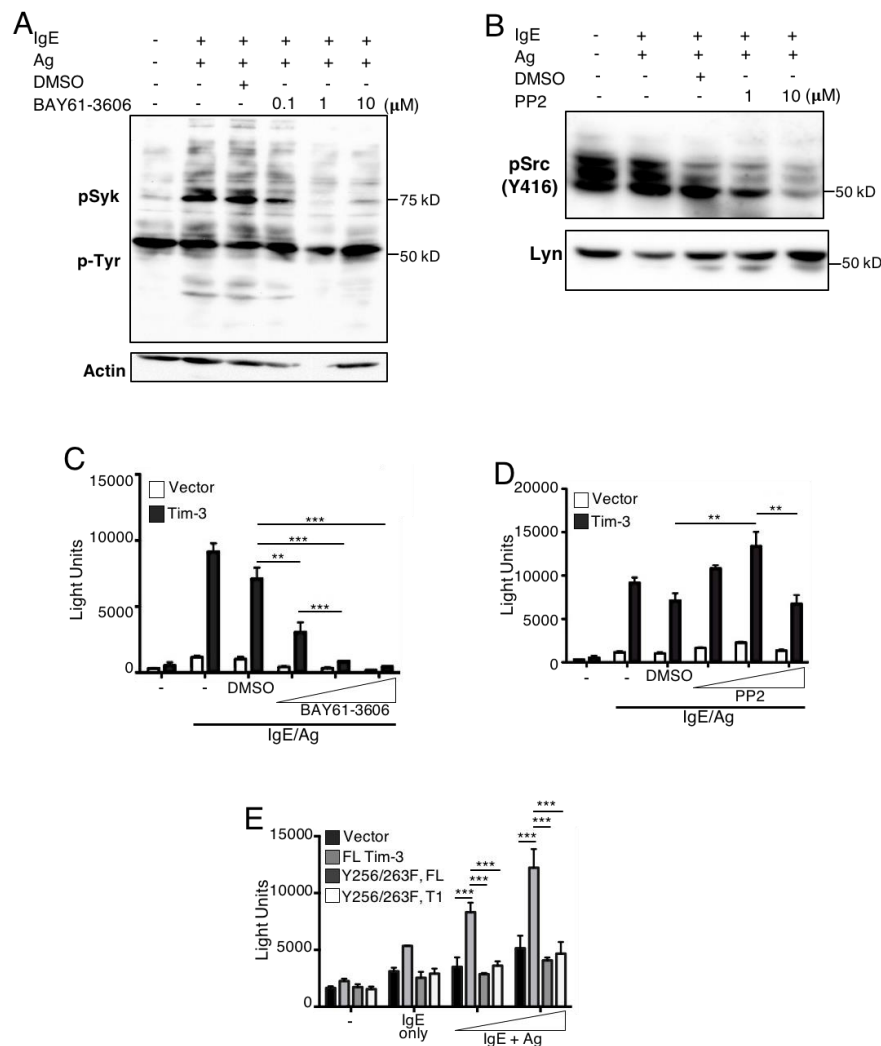


Figure 6-2: Tim-3 cytoplasmic tail tyrosines are required to augment IL-6 transcriptional activation, in a Syk-dependent manner. MC/9 mast cells were co-transfected with empty vector (pCDEF3) or full-length Flag-tagged Tim-3 and an IL-6 promoter luciferase reporter. Transfected cells were stimulated with IgE and Ag in the presence of vehicle control (DMSO), BAY61-3606 (C), or PP2 (D) compounds for six hours. Western blotting for effects of BAY61-3606 on total phosphotyrosine (A) and PP2 on pSrc (B) levels. MC/9 mast cells were transfected with the indicated Tim-3 constructs and IL-6 promoter luciferase reporter and stimulated as described above (E). Results are representative of three independent experiments performed in triplicate. ** $p < 0.005$, *** $p < 0.0005$.

6.4 DISCUSSION

FcεRI is a multi-chain receptor lacking intrinsic kinase activity and as such employs Src family tyrosine kinases (SFKs) for signal initiation and propagation. We showed that the tyrosine-containing cytoplasmic tail of Tim-3, which is indispensable for Tim-3 co-stimulation of T cell activation (308), was also required for its co-stimulatory activity in mast cells. NF-κB and NFAT/AP1 promoter activity were most affected when five of the six tyrosines were removed. While it is possible that Tim-3 might signal through a tyrosine-independent mechanism, we consider this unlikely, given the lack of any other obvious signaling motifs in this region and our previous findings that Fyn kinase could associate and phosphorylate Tim-3 at these residues (308). Similar to transient expression on T cells, we did not observe further augmentation by Tim-3 upon cross-linking by Flag antibody, further supporting our previous findings that Tim-3 expression alone was sufficient to drive Ag-mediated transcriptional response. The Tim-3 ligand galectin-9 is expressed on mast cells, and phosphatidylserine (PS) is present in the system as a result of electroporation-induced cell death. However, the enhancement observed here does not appear to be due to galectin-9 or PS interaction since endogenous Tim-3 is present on MC/9 yet does not contribute to any significant transcriptional activation. Tim-3 ectopic expression slightly enhanced NF-AT reporter activity in IgE-sensitized cells, suggesting Tim-3 may contribute to an IgE-dependent antigen-independent signaling pathway to synergize with antigen stimulation for NF-AT activation.

We explored the contribution of SFKs involved in mast cell activation in an IL-6 reporter assay using the pan-SFK inhibitor PP2. Surprisingly, Tim3-mediated IL-6 reporter activity was not affected by high concentrations of PP2 and was in fact enhanced at low concentrations of PP2.

By contrast, pharmacologic inhibition of Syk drastically diminished Tim-3-mediated IL-6 reporter activity. These results indicate that Tim-3 activity converges on FcεRI-mediated intracellular pathways that require Syk for both of its catalytic and adaptor functions. Given the complex hierarchical network of multiple SFKs in mast cell regulation, their variable sensitivity to PP2, and the existence of both positive and negative regulatory activities depending on Ag affinity and concentration (109, 114, 302), it will be important to determine the prominent kinase(s) that cooperate with Tim-3 to amplify responses to Ag stimulation. Lyn is a particularly appealing candidate for this activity. Thus, Lyn has been shown to possess both positive and negative regulatory functions in FcεRI activation in response to intensity of stimulus (109), and recruitment and activation of Syk requires phosphorylation of FcεRIγ ITAMs by Lyn (101, 352).

Previous studies also showed that Lyn is constitutively associated with FcεRIβ in both soluble and lipid raft fractions and that this association is increased upon FcεRI aggregation (114). Thus, the majority of the evidence points to a close association between FcεRIβ, Lyn and Tim-3 under basal conditions. Upon high intensity Ag stimulation, FcεRI is then recruited into the lipid rafts, where enhanced β phosphorylation by Lyn occurs, to facilitate binding of phosphatases, leading to down-regulation of signaling (114). Coupled with our findings that Tim-3 cytoplasmic tyrosines are required for its function, Tim-3 interaction with the FcεRI and its associated SFKs as a result of its cytoplasmic tail phosphorylation is a potential mechanism worth exploring.

7.0 TIM-3 SHARES SIGNALING COMPONENTS WITH ANTIGEN RECEPTOR PATHWAY

7.1 INTRODUCTION

We wanted to further explore the possibility that Tim-3 could augment FcεRI signaling, as opposed to acting through a parallel signaling pathway. We generated BMMCs from transgenic Nur77^{GFP} mice (353), to determine if antibody-modulated Tim-3 activity could supplement Ag receptor activation. Nur77, also known as NR4A1, is a member of the orphan nuclear receptor subfamily NR4A, and is an immediate early gene that can be induced upon a variety of stimuli (354-356). In the mast cells, expression of the NR4A subfamily can be induced upon exposure to live streptococci, antigen cross-linking of the IgE receptor, and calcium ionophore (357). While NR4A1 is phosphorylated upon mast cell activation, little is known about the precise function of NR4A1 in mast cell biology (357). Nur77^{GFP} reporter mice have been useful tools to study development and activation of lymphocytes, as the intensity of T cell and B cell antigen receptor activation is proportional to GFP expression (353, 358). In this study, using two independently generated Nur77^{GFP} mouse models, we report for the first time the signaling pathways leading to Nur77 expression upon FcεRI activation. We also determine if Tim-3 engagement contributes to FcεRI signal intensity. Overall, our results reveal that Tim-3 acts at the proximity of antigen receptor activation to enhance mast cell activation.

7.2 MATERIALS AND METHODS

7.2.1 Mice

The Nur77^{GFP} reporter mouse, C57BL/6-Tg(Nr4a1-EGFP/cre)820Khog/J, referred to as Nur77-EGFP/Cre, was purchased from the Jackson Laboratory and maintained in the University of Pittsburgh Animal Facility.

7.2.2 Antibodies and reagents

Monoclonal anti-dinitrophenyl (DNP) antibody, IgE isotype, clone SPE-7, DNP₃₂-HSA, and cyclosporin A (CsA) were from Sigma-Aldrich (St. Louis, MO). DNP₅-BSA was purchased from Biosearch Technologies (Petaluma, CA). Purified polyclonal antibody (Tim-3 pAb) and normal goat IgG control were from R&D Systems (Minneapolis, MN). Recombinant Tim4-Fc was from Vijay Kuchroo (Harvard). Phorbol myristate acetate (283), ionomycin, inhibitors to Src-family kinases (PP2), Syk (BAY61-3606), PI3K (LY294002), MEK (UO126), and Akt (Akti 1/2) were purchased from Calbiochem/EMD Biosciences (San Diego, CA). Inhibitor to PKC (Bisindolylmaleimide (BIM) VIII) was from Cayman Chemical (Ann Arbor, MI).

7.2.3 BMMC isolation and culture

Bone marrow from an independently generated Nur77^{GFP} mouse model, Nur77-EGFP, was obtained from Arthur Weiss (University of California San Francisco). Nur77^{GFP} bone marrow cells were cultured in RPMI 1640 supplemented with 10% BGS, non-essential amino acid, 2-ME,

HEPES, pen/strep-glutamine, and 20% IL-3-conditioned media for 4-6 weeks, after which >90% of viable cells are mature mast cells (c-kit⁺ FcεRI⁺ by flow cytometry).

7.2.4 BMMC stimulation and flow cytometry

BMMCs were sensitized overnight with 1 µg/ml IgE in complete media with IL-3. Cells were then stimulated with DNP₃₂-HSA or DNP₅-BSA, with either goat isotype, Tim3 pAb, human IgG, or Tim4-Fc for indicated amount of time. For experiments involving inhibitor treatment, the indicated inhibitors or DMSO as vehicle control were added to the cell culture at the time of antigen stimulation for six hours. Cells were then analyzed for GFP expression by flow cytometry.

7.2.5 Statistical analysis

All statistical analysis was performed using Prism software (GraphPad Software). Paired, two-tailed Student's t-test, one-way and two-way ANOVA with Bonferroni post hoc test were used for data analysis and determination of p values, as appropriate.

7.3 RESULTS

7.3.1 Tim-3 cross-linking enhances intensity of FcεRI signaling in two separate Nur77^{GFP} mouse models

In the first mouse model, Nur77-EGFP (353), we found that Nur77^{GFP} expression could be induced in Ag-stimulated BMMCs in a dose-dependent manner (Fig. 7-1A) and its expression was maintained for at least 48 hours (Fig. 7-1B-D). Consistent with findings in T and B cells, cytokine stimulation by IL-3 alone did not drive Nur77^{GFP} expression. On the other hand, c-kit receptor engagement by stem cell factor (SCF) could activate Nur77 transcription (Fig. 7-1B). Nur77^{GFP} induction was further augmented in Tim-3 pAb co-stimulated cells (Fig. 7-1E-F), but not when Tim-3 pAb was used alone or administered with a low dose of either the phorbol ester PMA or ionomycin (Fig. 7-1H-I). The co-stimulatory effects of Tim-3pAb treatment was further highlighted since it could enhance both optimal and sub-optimal antigen concentrations, 50 and 10 ng/ml respectively (Fig. 7-2A-B). Our results further revealed that the co-stimulatory effect observed with Tim-3 pAb is unique to Tim-3, as engagement of Tim-1, another family member expressed on BMMCs, did not yield similar GFP up-regulation (Fig. 7-1G). Thus, our results demonstrate that Tim-3 acts at point proximal to FcεRI to up-regulate the magnitude of IgE/Ag-dependent signaling, rather than signaling through a parallel pathway.

We confirmed FcεRI-dependent Nur77 expression in the independently generated Nur77^{GFP} mouse model Nur77-EGFP/Cre (358) (Fig. 7-3A-B). In addition, we observed that antigen valency could modulate antigen receptor signal strength as the low valency antigen (DNP₅) induced higher level of GFP expression compared to high valency antigen (DNP₃₂) (Fig. 7-3C). Interestingly, we observed a different pattern of Nur77^{GFP} expression compared to those in

BMMCs generated from Nur77-EGFP. Specifically, antigen stimulation in Nur77-EGFP BMMCs induced a shift in GFP expression that reflected both increase in cell frequency and GFP intensity while the shift in Nur77-EGFP/Cre BMMCs was more uniform and reflected only increase in GFP expression (Fig. 7-1A and 7-3A). Similarly, Nur77^{GFP} intensity by Tim-3 pAb and antigen co-engagement reflected the patterns of the corresponding mouse models (Fig. 7-1E, 7-2A-B, and 7-3D). These results suggest that antigen receptor signaling can be modulated quantitatively and qualitatively, and Tim-3 can contribute to FcεRI in both ways.

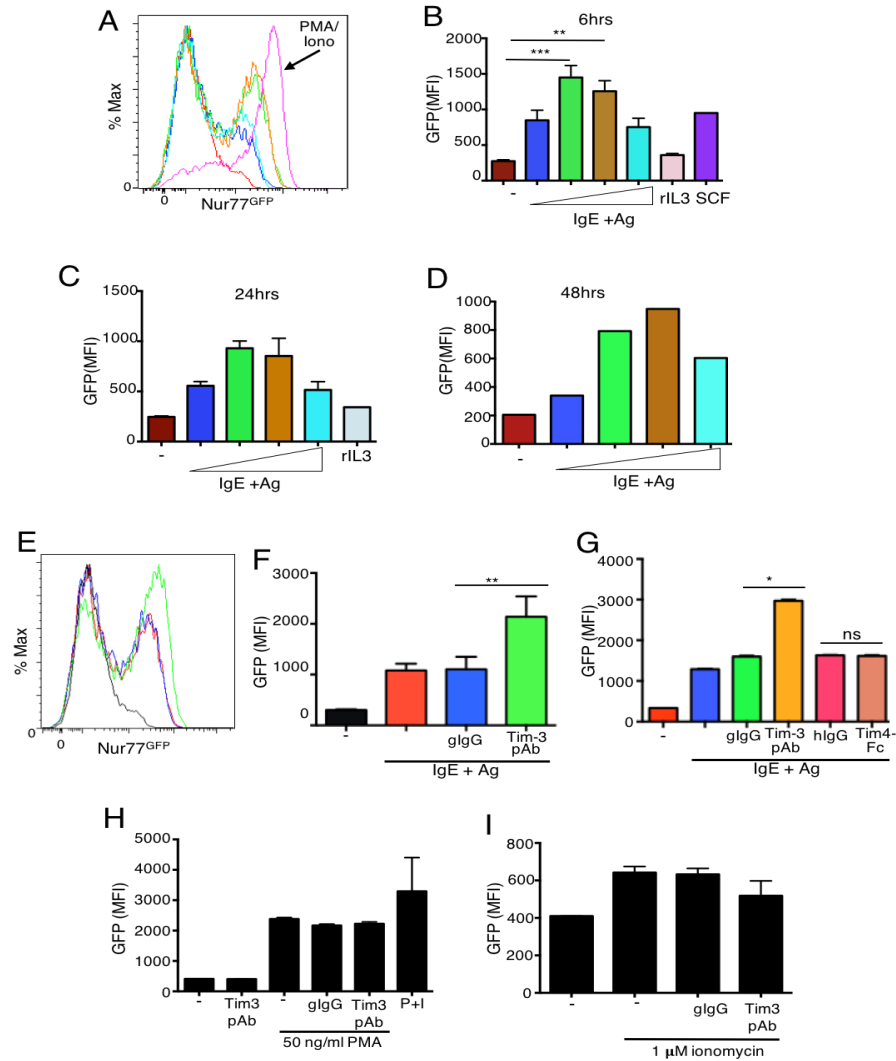


Figure 7-1: Nur77-GFP reporter expression is induced by IgE/Ag and enhanced by co-engagement of Tim-3. BMMCs were generated from Nur77-EGFP reporter mice, sensitized with IgE overnight and stimulated with antigen, 5 ng/ml of rIL-3 or SCF. (A-D) Antigen (DNP₃₂-HSA) was titrated over a range of 10-500 ng/ml. PMA plus ionomycin stimulation was used as a positive control. GFP expression was determined after stimulation for six (A-B), twenty-four (C), and forty-eight (D) hours. (E-F) Nur77^{GFP} BMMCs were stimulated with a fixed concentration of antigen, with or without Tim-3 pAb for six hours. (G) Nur77^{GFP} BMMCs were stimulated with IgE/Ag and either Tim-3 pAb or Tim4-Fc (which binds to Tim-1), or appropriate isotype controls. Results are the average of three independent experiments (A-C, E-G), two experiments (H-I) performed in duplicate, and once for Fig. 7-1D. *p < 0.05, **p < 0.005, ***p < 0.0005.

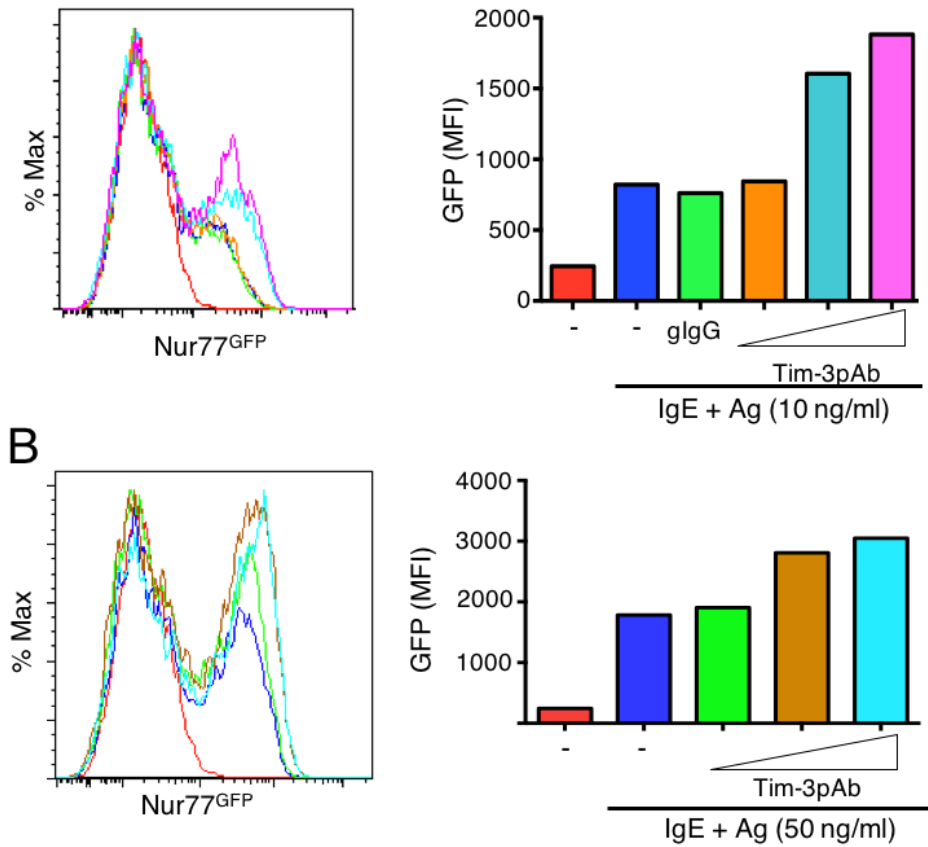


Figure 7-2: Tim-3 enhances IgE/Ag-mediated Nur77^{GFP} reporter expression in a dose dependent manner. Nur77^{GFP} BMMCs were sensitized with IgE and stimulated with 10 or 50 ng/ml DNP₃₂-HSA, isotype control or varying amounts of Tim3pAb for six hours. GFP was determined by flow cytometry.

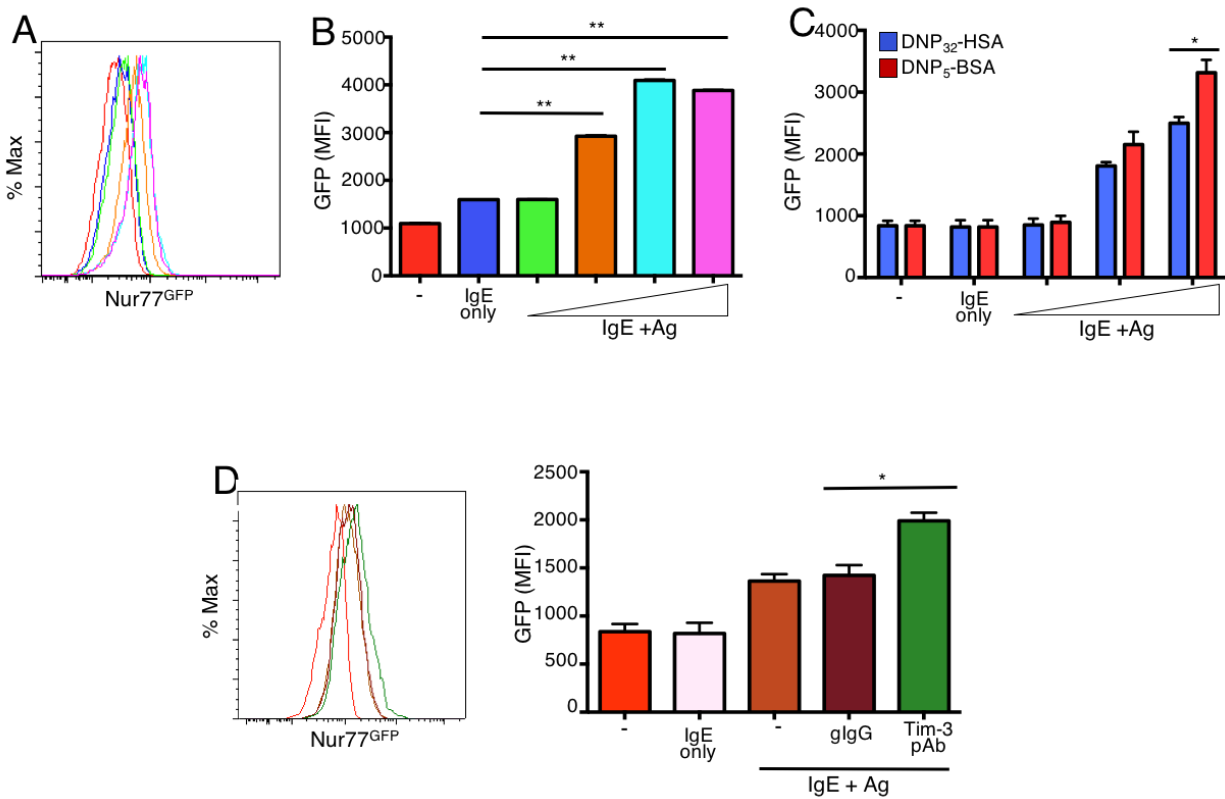


Figure 7-3: Tim-3 ligation augments antigen-induced Nur77^{GFP} expression in an independently generated Nur77^{GFP} mouse model. BMMCs were generated from Nur77-EGFP/Cre reporter mice, sensitized with IgE overnight and stimulated with increasing amount DNP₃₂-HSA or DNP₅-BSA for six hours prior to analysis of GFP expression by flow cytometry. (A-B) Antigen (DNP₃₂-HSA) was titrated over a range of 10-500 ng/ml. (C) Increasing concentrations of DNP₃₂-HSA or DNP₅-BSA were used for BMMC stimulation. (D) Tim-3 pAb or isotype control was administered with 50 ng/ml DNP₃₂-HSA. Results are average of three (A-B, D) and two (C) independent experiments performed in duplicates. * $p < 0.05$.

7.3.2 Signaling pathways that mediate activation of Nur77 expression downstream of FcεRI in BMMCs

Nur77^{GFP} has been demonstrated to be a faithful reporter for antigen receptor signaling in T and B cells, and now from our findings, in mast cells as well. Given the limited knowledge of how Nur77 expression is regulated in mast cells, we explored the contribution of various signaling pathways downstream of FcεRI to Nur77^{GFP} expression using pharmacologic inhibitors in both mouse models. Similar to our observations with IL-6 reporter activity (Fig. 6), low doses of the Src kinase inhibitor PP2 actually led to an Ag-dependent increase in Nur77^{GFP} induction, while high dose PP2 had no effect (Fig. 7-4A, C). By contrast, the Syk inhibitor completely abrogated Ag-stimulated Nur77 expression, a phenomenon comparable to the effects of SFK and Syk inhibitors on Nur77^{GFP} induction in B cells (Fig. 7-4A, C, and (353)). In addition, Nur77 expression was also regulated by MEK, PI3K/Akt, and calcineurin/NFAT (Fig. 7-4B, D). PKC inhibition led to a more significant induction of Nur77 expression compared to vehicle control in the Nur77-EGFP/Cre BMMCs, suggesting BMMCs from this strain may be more sensitive to PKC inhibition (Fig. 7-4B, D). Thus, based on results obtained with two independent Nur77^{GFP} reporter strains, we propose that Tim-3 functions to strengthen FcεRI-dependent signaling itself, and not through a parallel pathway.

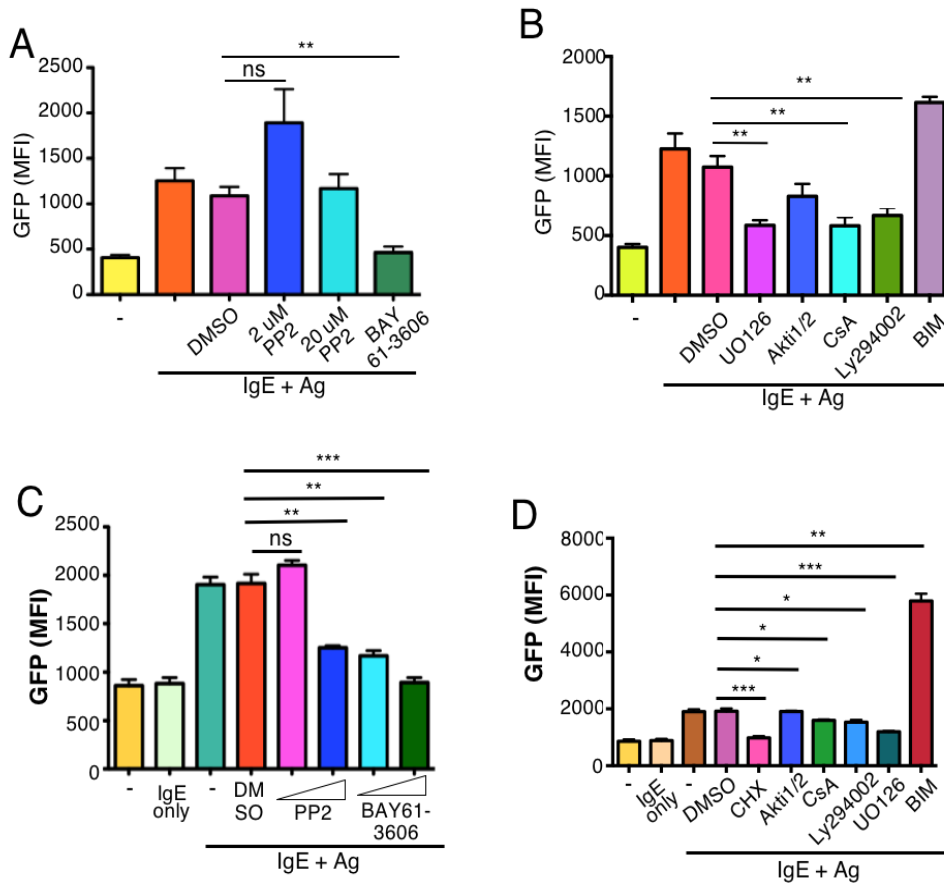


Figure 7-4: Fc ϵ RI-induced Nur77 activation is dependent on Syk and mediated by NFAT, PI3K/Akt, and MEK/ERK pathways. (A-B) Nur77^{GFP} BMMCs from Nur77-EGFP (A-B) or Nur77-EGFP/Cre (C-D) BMMCs were stimulated with IgE/Ag plus indicated inhibitors to Src kinases (PP2), Syk (BAY61-3606), MEK (UO126), Akt (Akt1/2), calcineurin (CsA), PI3K (LY294002) or PKC (BIM). GFP signal was quantified by flow cytometry. Results are the average of five independent experiments performed in duplicate. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

7.4 DISCUSSION

Using BMMCs generated from Nur77^{GFP} reporter mice (Nur77-EGFP), we show for the first time that Nur77^{GFP} expression can be used as a read-out for FcεRI signaling intensity. Similar to T and B cell receptor signaling (353), we observed a bimodal distribution of GFP in Ag-stimulated BMMCs, with increasing antigen dosage favoring an increase in the frequency of mast cells expressing Nur77^{GFP}, rather than just the amount of GFP itself. The same trend was also observed after co-stimulation with Tim-3 pAb. In another Nur77^{GFP} reporter mouse (Nur77-EGFP/Cre), while we only detected an increase in intensity of Nur77^{GFP} expression in response to antigen stimulation in mast cells, Tim-3 cross-linking also induced significant co-stimulation of FcεRI-mediated Nur77^{GFP} expression. Thus, Tim-3 engagement acts in a similar manner to increasing antigen dose, leading to intensified IgE receptor activation and an increase in the number of cells reaching a critical threshold for activation.

To better define which antigen receptor-induced signaling pathways were driving Nur77^{GFP} expression in mast cells, we employed various pharmacologic inhibitors. Our results revealed that, similar to antigen receptor signaling in B cells, Syk activity is required for *Nur77* transcription in mast cells, while calcineurin, PI3K/Akt and MAPK are less essential. In contrast to T cells where PKC is required for Nur77^{GFP} expression, treatment of mast cells with the pan-PKC inhibitor BIM actually up-regulated GFP expression. Given the differential expression of various PKC species in T cells and mast cells, BIM may have prevented the activation of a particular PKC isoform that is normally involved in negative regulation of Nur77 expression. One candidate for this activity is PKC δ, which was previously shown to act as a negative regulator of mast cell activation (340).

8.0 TIM-3 ASSOCIATES WITH ANTIGEN RECEPTOR PROXIMAL SIGNALING COMPLEX

8.1 INTRODUCTION

Binding of IgE Fc ϵ RI α chain stabilizes and increases surface expression of Fc ϵ RI, as well as maintains baseline protein synthesis of the receptor components (359). Receptor proximal signaling occurs when antigen cross-linking of pre-bound IgE induces a series of phosphorylation events on the signal amplifier Fc ϵ RI β chain and the signal transducer γ chain by Lyn kinase as well as recruitment of Syk (101-104). Lyn-mediated activation of Syk leads to phosphorylation and recruitment of molecular adaptors and secondary messengers generated by aggregation of surface receptors (Rivera, 2002). Our findings thus far suggest that Tim-3 partners closely with Fc ϵ RI and may modulate activity of Fc ϵ RI components.

In this study, we determined whether Tim-3 expression is regulated by mast cell activation, and how Tim-3 ligation may affect Ag-induced Fc ϵ RI internalization, a hallmark of receptor signaling downregulation (360). Here we show that Tim-3 expression is co-modulated with Fc ϵ RI upon antigen aggregation while Tim-3 engagement does not alter receptor surface expression or internalization. In addition, we observe constitutive association of Tim-3 with Fc ϵ RI β and γ subunits. Finally, we examine the presence of the proposed Tim-3 regulators Bat3 and CEACAM1 on mast cells and show that the co-stimulatory activity of Tim-3 in mast cells is independent of Bat3 and CEACAM1.

8.2 MATERIALS AND METHODS

8.2.1 BMMC isolation and cell line culture

C57BL/6 purchased from Jackson Laboratory (Bar Harbor, ME) were cultured in RPMI 1640 supplemented with 10% BGS, non-essential amino acid, 2-ME, HEPES, pen/strep-glutamine, and 20% IL-3-conditioned media for 4-6 weeks, after which >90% of viable cells are mature mast cells (c-kit⁺ FcεRI⁺ by flow cytometry). MC/9 mast cells were maintained in DMEM supplemented with 10% BGS, 2-ME, pen/strep with glutamine, and 10% IL3-conditioned media.

8.2.2 Antibodies and reagents

Monoclonal anti-dinitrophenyl (DNP) antibody, IgE isotype, clone SPE-7, anti-Flag antibody M2, and DNP₃₂-HSA were from Sigma-Aldrich (St. Louis, MO). Phorbol myristate acetate (283), ionomycin, aprotinin, leupeptin, pepstatin, sodium orthovanadate, and 4-(2-aminoethyl)benzene sulfonyl fluoride (AEBSF) were purchased from Calbiochem/EMD Biosciences (San Diego, CA). Purified polyclonal antibody (Tim-3 pAb), directly fluorescent conjugated antibody to murine Tim-3, and normal goat IgG as isotype control were from R&D Systems (Minneapolis, MN). mAb to Tim-3 5D12 was from Vijay Kuchroo (Harvard Medical School). Anti-IgE-FITC antibody was from Biolegend (San Diego, CA). Antibody to FcεRI β chain was from Santa Cruz Biotechnology (Dallas, TX). Antibodies to Bat3 and FcεRI γ chain were from EMD Millipore (Billerica, MA). Fluorescent antibodies to CEACAM1/2 (CD66a) and mouse IgG isotype control were obtained from eBioscience (San Diego, CA).

8.2.3 BMMC stimulation for modulation of Tim-3 and CEACAM1 expression

BMMCs were sensitized with 1 $\mu\text{g/ml}$ IgE overnight and stimulated with 50 ng/ml DNP₃₂-HSA for the indicated times. Cells were fixed with 1.5 % PFA and permeabilized with ice cold methanol prior to staining for total Tim-3 and flow cytometry. Surface Tim-3 and CEACAM1 was also determined by flow cytometry.

8.2.4 Western blotting and immunoprecipitation

BMMCs and MC/9 were sensitized with 0.5 $\mu\text{g/ml}$ IgE for two hours at 37°C in Tyrode's buffer. Cells were then stimulated with DNP₃₂-HSA for indicated times in Tyrode's buffer. Cells were lysed in 1% Nonidet-P-40 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) supplemented with AEBSF, aprotinin, leupeptin, pepstatin, sodium fluoride, sodium orthovanadate, and beta-glycerophosphate on ice for twenty minutes. Lysates were centrifuged at 4°C for fifteen minutes. For western blotting analysis, SDS-containing sample buffer was added to lysates prior to loading onto 10% or 12% SDS-PAGE gels when analyzing for Fc ϵ RI β and γ chains. For IPs, lysates were incubated overnight at 4°C with appropriate antibodies. Immune complexes were precipitated at 4°C for two hours with protein A or G agarose beads (Pierce) for rabbit or mouse Abs respectively, then washed with NP-40 lysis buffer three times before analysis by SDS-PAGE gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using the PierceG2 fast blotter (Pierce), probed with appropriate antibodies, and imaged on a ProteinSimple Fluochem M cooled CCD imager (ProteinSimple).

8.2.5 FcεRI internalization assay

BMMCs were sensitized with 0.5 µg/ml IgE for two hours at 37°C in Tyrode's buffer. BMMCs were incubated with 100 ng/ml DNP₃₂-HSA for one hour on ice to cross-linked IgE-bound receptors without inducing internalization. Aliquots of Ag-bound cells were removed and placed in a 37°C water bath for indicated times to allow for receptor internalization. Surface Tim-3 and FcεRI were determined by staining with anti-Tim-3-PE and anti-IgE-FITC prior to flow cytometry analysis. %receptor internalization was calculated as $[\text{MFI}(\text{time } X) - \text{MFI}(\text{time } 0')]/\text{MFI}(\text{time } 0')$ where X indicates incubation time.

8.2.6 TIRF microscopy and analysis

Anti-DNP IgE was labeled with Alexa 647 (Life Technologies). BMMCs were transfected with Tim-3-mYFP construct by nucleofection (Lonza). Twenty-four hours post-transfection, BMMCs were sensitized with 1µg/ml IgE-Alexa 647 for one hour at 37°C. 3×10^5 cells were loaded into poly-D-lysine treated glass bottom dishes (321) that were coated with 1 mg/ml DNP₃₂-HSA antigen in PBS for one hour at 37°C and fixed with 2% PFA. Images were captured in TIRF mode using Nikon Eclipse Ti Live Cell Microscope equipped with Andor Zyla VSC-00311 camera and Apo TIRF 100x oil DIC N2 objective, and analyzed with NIS-Elements Ar 4.20 software (Nikon Instruments Inc.). Colocalization analysis was performed using the Spots Colocalize function of the Imaris Scientific 3D/4D Imaging Processing and Analysis software (Bitplane).

8.3 RESULTS

8.3.1 Tim-3 ligands Bat3 and CEACAM1 are expressed by mast cells and do not interfere with Tim-3 cross-linking

The human leukocyte antigen B (HLA-B)-associated transcript 3 (Bat3) has been proposed to be a negative regulator of Tim-3 and to protect Th1 T cells from Tim-3-mediated cell death and exhaustion (309). We therefore determined whether the co-stimulatory activity of Tim-3 in mast cells might be due to a lack of Bat3 expression. We found that Bat3 was expressed in both primary BMMCs and the MC/9 mast cell line and that its expression was not affected by FcεRI cross-linking, either alone or in conjunction with Tim-3 engagement (Fig. 8-1A-B). Recently, carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1), a molecule inhibitory activity, was described as a facilitator of Tim-3 surface expression and its subsequent inhibitory function in CD4⁺ T cells by forming heterodimers with Tim-3 (271). As with Bat3, we wanted to determine whether lack of CEACAM1 expression might explain the co-stimulatory activity of Tim-3 on mast cells. We found that CEACAM1 was indeed expressed at a high level on both the MC/9 mast cell line and primary BMMCs (Fig. 8-1C-D). While surface and intracellular Tim-3 expression was reduced upon Ag stimulation, CEACAM1 levels remained relatively unchanged (Fig. 8-1E-G). Thus, the ability of Tim-3 to act as a co-stimulatory molecule to enhance FcεRI-mediated mast cell activation is not due to an absence of Bat3 and/or CEACAM1 expression in these cells.

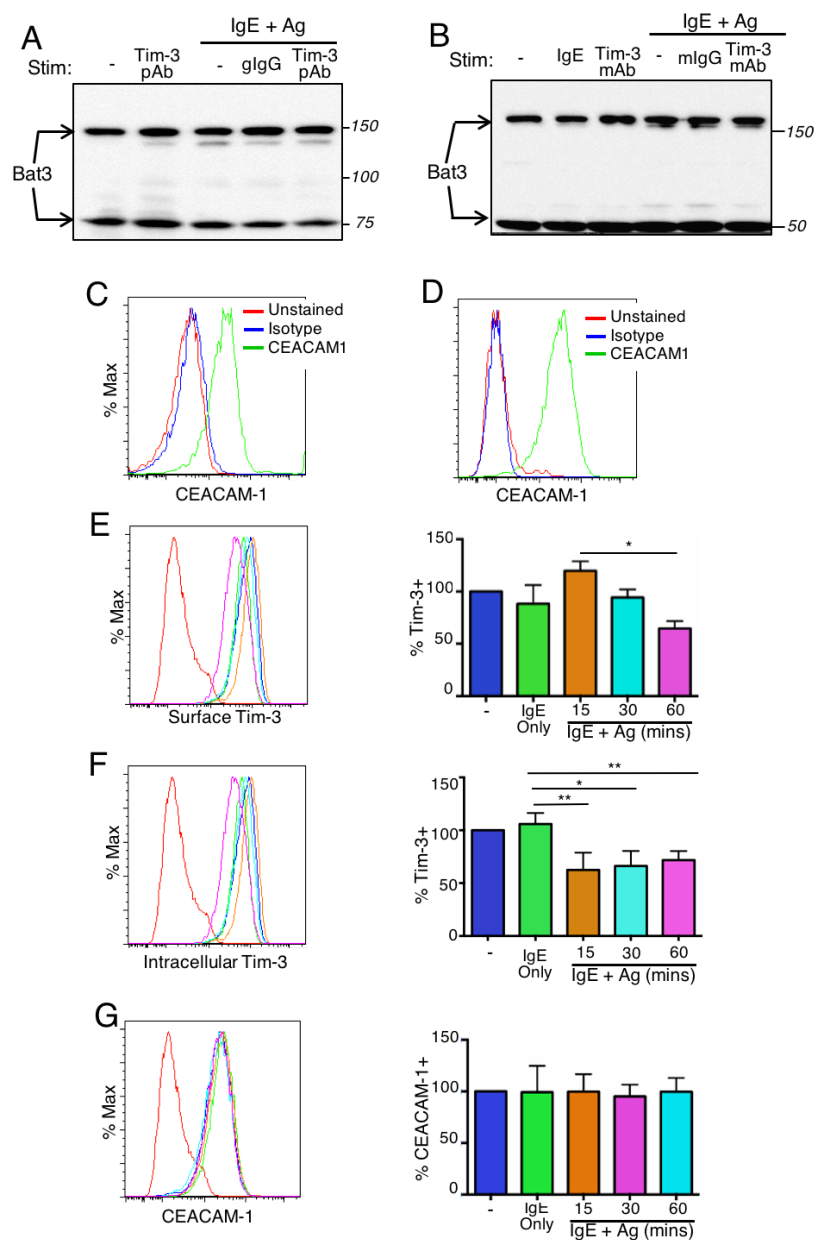


Figure 8-1: Tim-3 is co-modulated with FcεRI after stimulation with antigen and IgE. (A-D) Tim-3-interacting proteins Bat3 and CEACAM1 are expressed in mast cells. BMMCs were stimulated with IgE/Ag in the presence of isotype control or Tim-3 pAb. Bat3 expression on BMMC (A) and MC/9 (B) was determined by western blotting. Constitutive CEACAM1 surface expression on BMMC (C) and MC/9 (D) was determined by flow cytometry. CEACAM1 and Tim-3 expression on BMMC post-stimulation with IgE/Ag at indicated times were determined by flow cytometry (E-G). Results are representative of two independent experiments (A-D). Results are average of three independent experiments (E-G). *p < 0.05; **p < 0.05.

8.3.2 Antigen-mediated surface FcεRI internalization is not affected by Tim-3 ligation

Since antigen stimulation downregulated both surface and total Tim-3 expression, we determined if Tim-3 engagement could promote FcεRI surface expression or delay receptor internalization post antigen stimulation, both of which may facilitate extended and intensified FcεRI proximal signaling. As expected, longer IgE sensitization time led to increased surface expression of IgE-bound FcεRI, which was not augmented with Tim-3 pAb co-treatment (Fig. 8-2A). Similarly, we observed robust receptor internalization within five minutes of antigen cross-linking. However, additional Tim-3 pAb treatment did not alter the kinetics of FcεRI internalization (Fig. 8-2B). As a result, while Tim-3 expression is co-modulated by FcεRI activation, Tim-3 ligation does not appear to regulate the FcεRI itself, but rather its signaling activity.

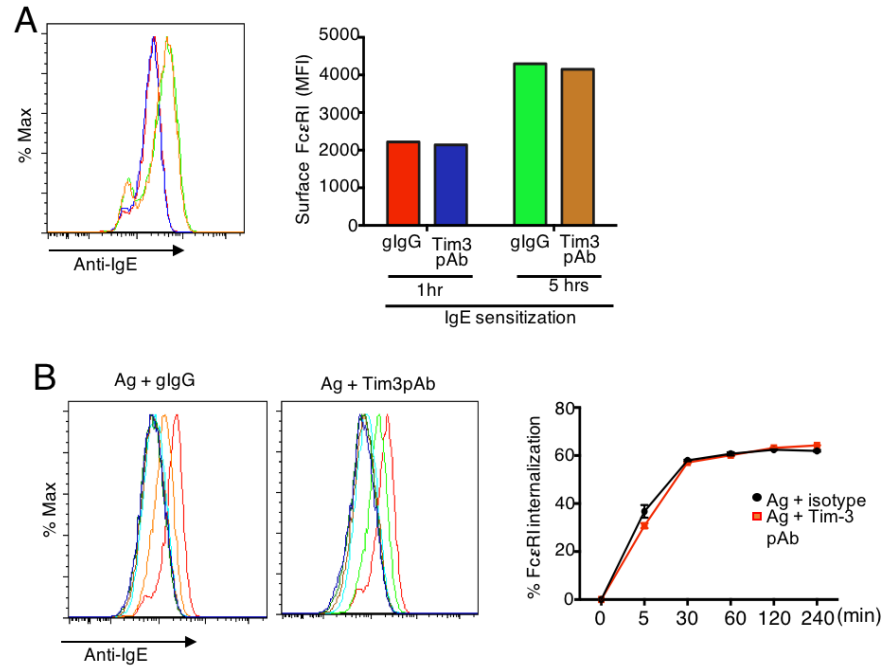


Figure 8-2: Antigen-mediated receptor internalization is not modulated by Tim-3 cross-linking. BMMCs were sensitized with IgE for the indicated times prior to determination of surface IgE-bound FcεRI by flow cytometry (A). BMMCs were sensitized with IgE for one hour and incubated with antigen on ice for one hour prior to receptor cross-linking for the indicated times (B). % FcεRI internalization was determined by flow cytometry. Results are representative of two independent experiments.

8.3.3 Tim-3 co-localizes with components of the FcεRI proximal signaling complexes

Consistent with the findings discussed above, which suggest that Tim-3 partners closely with the FcεRI, we observed by co-immunoprecipitation constitutive interaction between Tim-3 and the FcεRI α and γ subunits (Fig. 8-3A-B). These interactions were diminished somewhat after Ag stimulation, possibly due to turnover in the total amount of Tim-3 protein. Next we utilized total internal reflection fluorescence (TIRF) microscopy to obtain further evidence that Tim-3 localizes with, or proximal to, FcεRI. Thus, we were able to observe partial co-localization of Tim-3 and the FcεRI receptor at the cell surface when a Tim-3-mYFP construct was expressed in WT BMMCs (yellow clusters; Fig. 8-3C). Ligation of FcεRI by IgE/Ag complexes has been shown to trigger receptor endocytosis and subsequent ubiquitin-mediated degradation, resulting in signal termination (360). Since we observed a reduction of surface Tim-3 upon IgE/Ag stimulation (Fig. 8-2E), our data suggested that surface Tim-3 may be internalized by a similar mechanism, perhaps even as part of the same FcεRI complex. We also determined if Tim-3, due to its proximity to the FcεRI, could associate with Lyn that binds constitutively to FcεRI β . While we observed association in some experiments, the binding was not consistent, and thus will require further investigation.

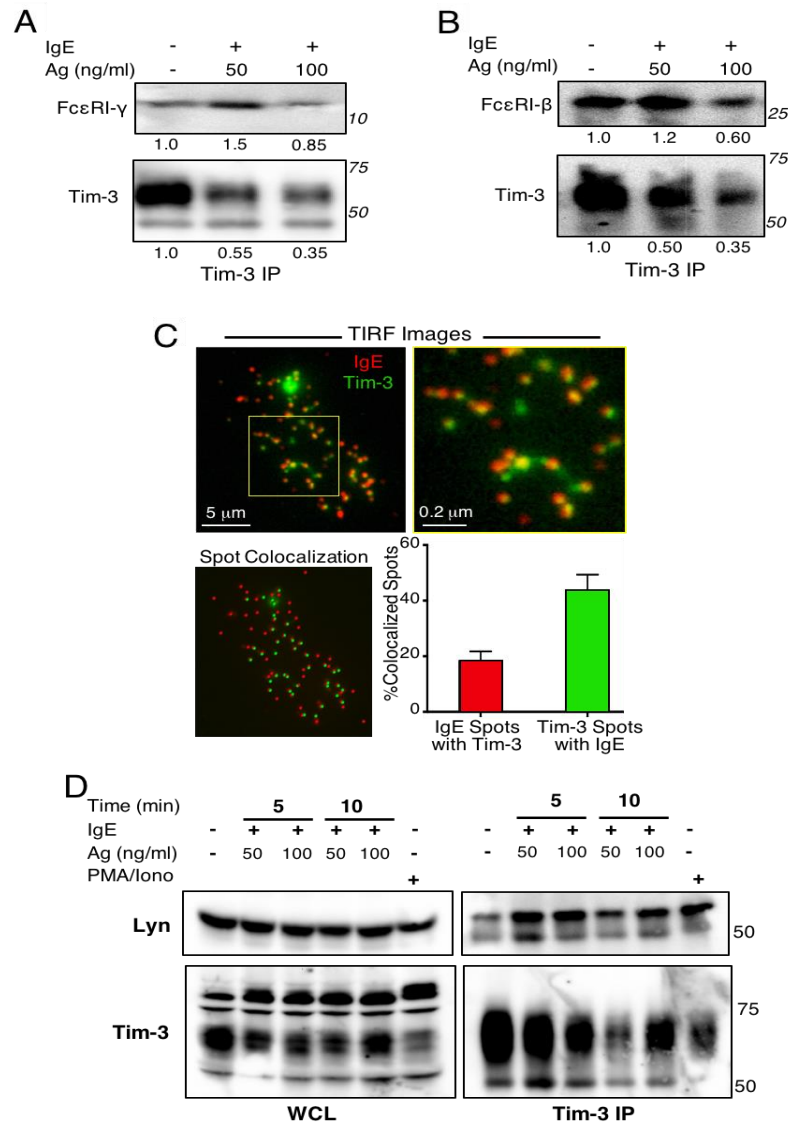


Figure 8-3: Tim-3 associates with components of the FcεRI signaling complexes. MC/9 were stimulated as indicated. Tim-3 IP's were analyzed for the presence of FcεRI γ chain (A), β chain (B) (upper panels), or Tim-3 (lower panels). (C) BMMCs were transfected with Tim3-mYFP, sensitized with IgE-Alexa 647 for one hour, and settled onto poly-D-lysine treated glass-bottom dishes coated with 1mg/ml DNP₃₂-HSA at 37°C. Cells were fixed with 2% PFA after one hour, and TIRF images were collected. The percentage of colocalized spots was derived from the average of twenty-one cells both labeled with anti-IgE-Alexa 647 and expressing Tim3-mYFP, in three independent experiments. (D) Lysates from MC/9 stimulated with IgE/Ag were subjected to Tim-3 immunoprecipitation and analyzed for the presence of Lyn (upper panels) and Tim-3 (lower panels). Results are representative of two independent experiments (A-B) and three out of seven experiments (D).

8.4 DISCUSSION

In addition to the previously described “in-trans” ligands of Tim-3, we also examined other effector molecules that have been described as regulators of Tim-3 function. We found that the chaperone Bat3, a putative negative regulator of Tim-3 function in T cells (309), is expressed in mast cells, but mechanisms of its function are relatively unknown. CEACAM1 was recently described as a binding partner of Tim-3 and was observed to be co-expressed with Tim-3 during the induction of T cell tolerance (271). We detected CEACAM1 expression in both the MC/9 mast cell line and primary BMMCs, although its role in mast cell function is unclear. Thus, the potential roles that Bat3 and CEACAM1 may play in regulating mast cell function via Tim-3 require further investigation. Nonetheless, the enhancement of mast cell activation by Tim-3 cannot be solely attributed to the lack of expression of either Bat3 or CEACAM1.

We observed constitutive association of Tim-3 with FcεRI β and γ subunits. While there was a slight increase of Tim-3 association to β and γ upon antigen stimulation, the slight decrease in associated β and γ was probably due to Tim-3 downregulation. In fact, antigen-induced turnover of surface and total Tim-3 has been consistently observed in both MC/9 and BMMCs (Fig. 8-1, 8-3). Thus, it is possible that Tim-3 is regulated by FcεRI cross-linking at the protein level, which further supports our hypothesis that Tim-3 is internalized and processed by the same mechanism as the FcεRI. To test this possibility, we performed TIRF microscopy to examine the potential interaction of Tim-3 with FcεRI on the cell surface without interference from the intracellular pool of Tim-3. We were not able to detect co-localization of Tim-3 and FcεRI at resting state as the mast cells did not attach and spread efficiently without antigen coating on the poly-D-lysine treated glass-bottom dishes. Since we could only image the cells that have properly attached to the plate

and cross-linked by antigen, it is possible that we only captured a fraction of the transected Tim-3 pool remaining on the cell surface by the end of stimulation. Nevertheless, we did observe co-localization of Tim-3 and IgE-bound receptor at the cell surface, suggesting Tim-3 not only shares signaling pathways but also physically associates with the FcεRI. Future studies will focus on methods to promote rapid and efficient cell adherence to the glass dishes so we can observe the pattern of Tim-3 expression on mast cells both at basal level and upon antigen cross-linking using live cell imaging.

We detected a certain level of Lyn association with Tim-3 at resting state and upon antigen stimulation. Previous studies also showed that Lyn is constitutively associated with FcεRIβ in both soluble and lipid raft fractions and that this association is increased upon FcεRI aggregation (114). Upon high intensity Ag stimulation, FcεRI is then recruited into the lipid rafts, where enhanced β phosphorylation by Lyn occurs, to facilitate binding of phosphatases, leading to down-regulation of signaling (114). Thus, the majority of the evidence points to a close association between FcεRIβ, Lyn and Tim-3 under basal conditions. Therefore, it is important to determine the distribution pattern of Tim-3 in both lipid raft and non-raft fraction under antigen stimulation, and if Tim-3 cross-linking will affect localization of Tim-3 or FcεRI β, leading to decreased association with phosphatases and enhanced positive antigen receptor signaling.

9.0 TIM-3 ENGAGEMENT ENHANCES ANTIGEN-INDUCED PHOSPHORYLATION OF PHOSPHOLIPASE C GAMMA AND RIBOSOMAL PROTEIN S6

9.1 INTRODUCTION

Antigen stimulation of FcεRI triggers activation of the Src kinase Lyn, which controls the primary pathway downstream of FcεRI. Receptor aggregation by Ag stimulation leads to Lyn-mediated phosphorylation of the FcεRI β chain, as well as phosphorylation of the γ subunit of the receptor, which promotes Syk recruitment and activation. Syk mediates phosphorylation of downstream adaptor molecules such as LAT and Slp76, and the enzyme PLC-γ1 (350, 351). Phosphorylation and activation of PLC-γ1 leads to generation of inositol triphosphate (IP₃) essential to calcium mobilization for degranulation and activation of NF-AT for cytokine production (157). DAG generated by PLC-γ1 can activate PKC, and contributes to Fos and Jun activation (166). Thus, PLC-γ1 is an important antigen receptor proximal signaling effector with multiple involvement in both the immediate and late-phase mast cell activation.

Syk-mediated phosphorylation of the adaptor molecule LAT leads to activation of the Ras/Raf/MAPKs pathways including ERK, p38, and JNK (162). These MAPKs go on to regulate activation of transcription factors Fos and Jun, component of the activating protein (AP)-1, NFAT, and NF-κB, and thus has a role in cytokine production (163-165). Together, the Lyn/Syk/LAT/PLCγ axis is responsible for initiating and maintaining FcεRI signaling required for mast cell effector function. In the following study, we systematically determine how Tim-3 ligation

modulates both receptor proximal and downstream signaling pathways leading to enhanced degranulation and cytokine production in antigen-activated mast cells. We show that Tim-3 pAb augments total tyrosine phosphorylation in antigen-stimulated BMMCs. While Syk and MAPKs activation is not affected, phosphorylation of PLC γ 1 and the mTOR substrate ribosomal S6 is enhanced by Tim-3 pAb co-stimulation, thus providing a potential mechanism for augmented cytokine production.

9.2 MATERIALS AND METHODS

9.2.1 Mice and BMMC culture

The Nur77^{GFP} reporter mouse, C57BL/6-Tg(Nr4a1-EGFP/cre)820Khog/J, was purchased from the Jackson Laboratory and maintained in the University of Pittsburgh Animal Facility. C57BL/6 mice (7-8 weeks old) purchased from the Jackson Laboratory (Bar Harbor, ME). Bone marrow cells were cultured in RPMI 1640 supplemented with 10% BGS, non-essential amino acid, 2-ME, HEPES, pen/strep-glutamine, and 20% IL-3-conditioned media for 4-6 weeks, after which >90% of viable cells are mature mast cells (c-kit⁺ Fc ϵ RI⁺ by flow cytometry).

9.2.2 Antibodies and reagents

Monoclonal anti-dinitrophenyl (DNP) antibody, IgE isotype, clone SPE-7, and DNP₃₂-HSA, were from Sigma-Aldrich (St. Louis, MO). Purified polyclonal antibody (Tim-3 pAb) and normal goat IgG control were from R&D Systems (Minneapolis, MN). Phospho-specific Ab's PLC- γ 1 (Y783)

and total PLC- γ 1 were obtained from BD Biosciences (San Jose, CA). Phospho-specific antibodies to Syk (519/520), p38 MAPK (T180/Y182), SAPK/JNK (T183/Y185), S6(S235/234), and ERK (T202/Y204) were from Cell Signaling Technology (Danvers, MA). Antibody against total tyrosine phosphorylation (clone 4G10) was from EMD Millipore (Darmstadt, Germany).

9.2.3 BMMC stimulation and Western blotting

BMMCs were sensitized with IgE overnight and stimulated with DNP₃₂-HSA with either goat isotype or Tim-3 pAb for the indicated time. Cells were lysed in 1% Nonidet-P-40 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) supplemented with AEBSF, aprotinin, leupeptin, pepstatin, sodium fluoride, sodium orthovanadate, and beta-glycerophosphate on ice for twenty minutes. Lysates were centrifuged at 4°C for fifteen minutes. For western blotting analysis, SDS-containing sample buffer was added to lysates prior to loading onto 10% SDS-PAGE gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using the PierceG2 fast blotter (Pierce), probed with appropriate antibodies, and imaged on a ProteinSimple Fluochem M cooled CCD imager (ProteinSimple).

9.2.4 BMMC stimulation and phospho-flow cytometry

BMMCs were sensitized with IgE overnight and stimulated with DNP₃₂-HSA either with goat isotype or Tim-3 pAb for indicated times. Cells were fixed with 1.5% PFA and permeabilized with ice cold methanol prior to staining for phospho-specific Ab's to Syk (Y519/520), ERK (T202/Y204), and S6 (S235/236) and flow cytometry analysis.

9.3 RESULTS

9.3.1 Tim-3 enhances Ag-triggered phosphorylation of PLC- γ 1

Our findings thus far demonstrated that Tim-3 expression co-stimulated NF- κ B and NF-AT/AP1, leading to IL-6 transcriptional activation and cytokine production, possibly by augmenting antigen receptor signaling. To determine the molecular mechanisms of Tim-3 signaling in mast cells, we first examined whether Tim-3 ligation could enhance total tyrosine phosphorylation. Consistent with our cytokine and Nur7^{GFP} data, Tim-3 pAb alone could not stimulate mast cell activation (Fig. 9-1A). Compared to antigen alone, addition of Tim-3 pAb induced stronger intensity of multiple bands ranging from 25kD to 100kD (Fig. 9-1A). The double-band at 50kD is characteristic of Src family kinase phosphorylation, and is not significantly modulated by Tim-3 activity. We were particularly interested in the bands at approximately 75kD and 100kD as they correlate with the size of Syk and PLC γ respectively. Subsequently, robust Syk phosphorylation at its auto-phosphorylation sites, tyrosines 519 and 520 critical to Syk activity (131, 134-136) was detected two minutes post stimulation. However, Tim-3 cross-linking did not modify phosphorylation of these sites on Syk (Fig. 9-1B). On the other hand, phosphorylation of PLC- γ 1 (at Y783) was more robust in BMMCs co-stimulated with Ag and Tim-3 pAb (Fig. 9-1B), compared with cells stimulated with Ag alone. Therefore, Tim-3 may enhance Ag-mediated NF-AT activation through activity of PLC γ in mast cells.

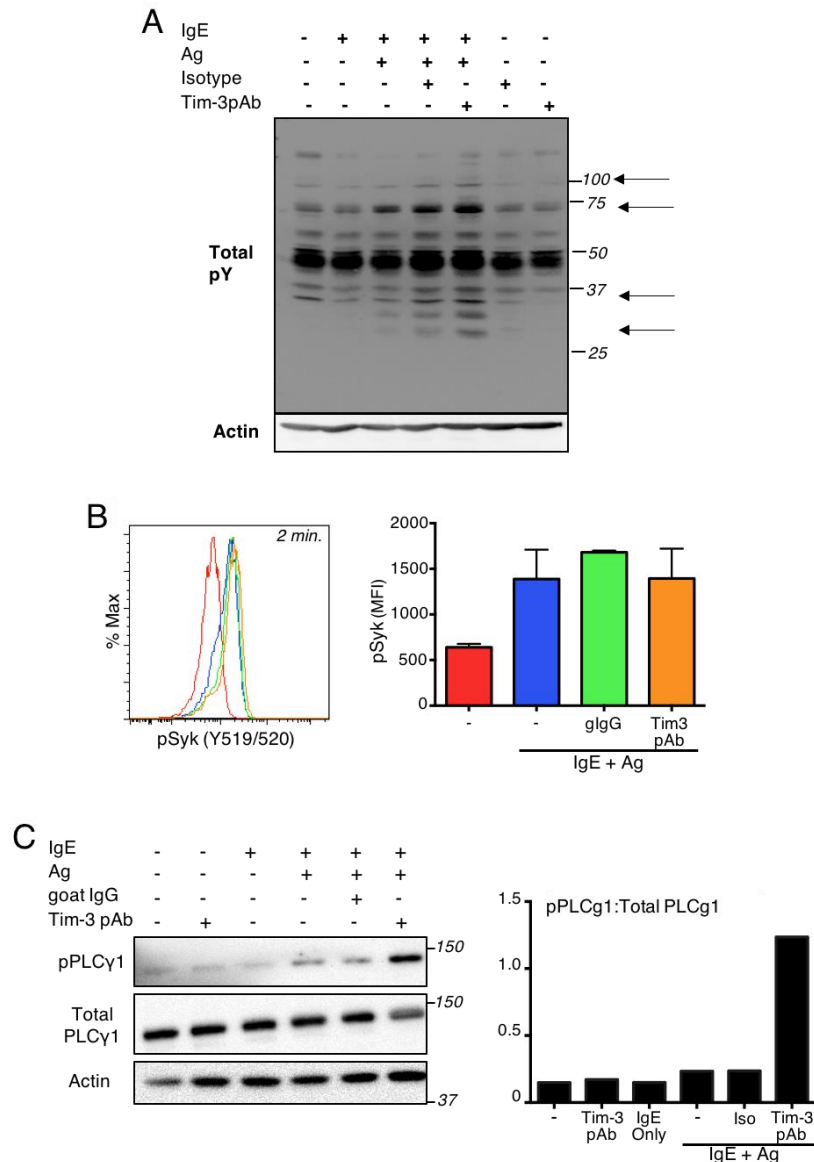


Figure 9-1: Tim-3 pAb co-stimulates Ag-mediated total tyrosine and PLC γ 1 phosphorylation. BMBCs were sensitized with IgE overnight and stimulated as indicated for ten minutes, lysed and analyzed by western blotting for total tyrosine phosphorylation (A). BMBCs were stimulated as described for two minutes prior to determination of pSyk (Y519/520) by flow cytometry (B). BMBCs were stimulated as indicated for ten minutes and analyzed for phospho-PLC- γ 1 (upper panel), total PLC- γ 1 (middle panel), and actin (C) by western blotting. Relative proportions of PLC- γ 1 phosphorylation are quantified in the right panel. BMBCs were stimulated as described for two minutes prior to determination of pSyk (Y519/520) by flow cytometry (B). Results are representative of two (A) and three (C) independent experiments, and average of three independent experiments (B).

9.3.2 Tim-3 engagement sustains FcεRI-mediated phosphorylation of ribosomal protein S6 but not MAPKs in BMMCs

Next we determined if Tim-3 is involved in regulation of the p38, JNK, and ERK MAPKs pathways. While Ag stimulation induced robust phosphorylation of all three MAPK pathways, Tim-3 pAb treatment did not exert any additional effects (Fig. 9-2A-C). Similarly, transient Tim-3 knockdown by siRNA did not alter Ag-mediated ERK phosphorylation, suggesting Tim-3 expression or activation is not involved in regulating the MAPK, particularly ERK, pathways (Fig. 9-2D).

One of the major signaling pathways that regulate cell growth, protein synthesis and metabolism in mast cell is the PI3K/mTOR pathway (361). We observed robust phosphorylation of ribosomal protein S6 after thirty minutes of IgE/Ag stimulation, when 80% of BMMCs exhibited high levels of pS6 and Tim-3 pAb did not have any additive effect (Fig. 9-2E). However, Tim-3 cross-linking was able to maintain a significantly larger proportion of pS6^{high} cells for as long as four hours after Ag stimulation, when pS6 levels had returned to baseline (Fig. 9-2E). More importantly, the increase in pS6 correlated with the intensity of FcεRI engagement, as shown with Nur77^{GFP} BMMCs (Fig. 9-2F). Overall, these results are consistent with a close physical and functional association between Tim-3 and the FcεRI, as also shown above.

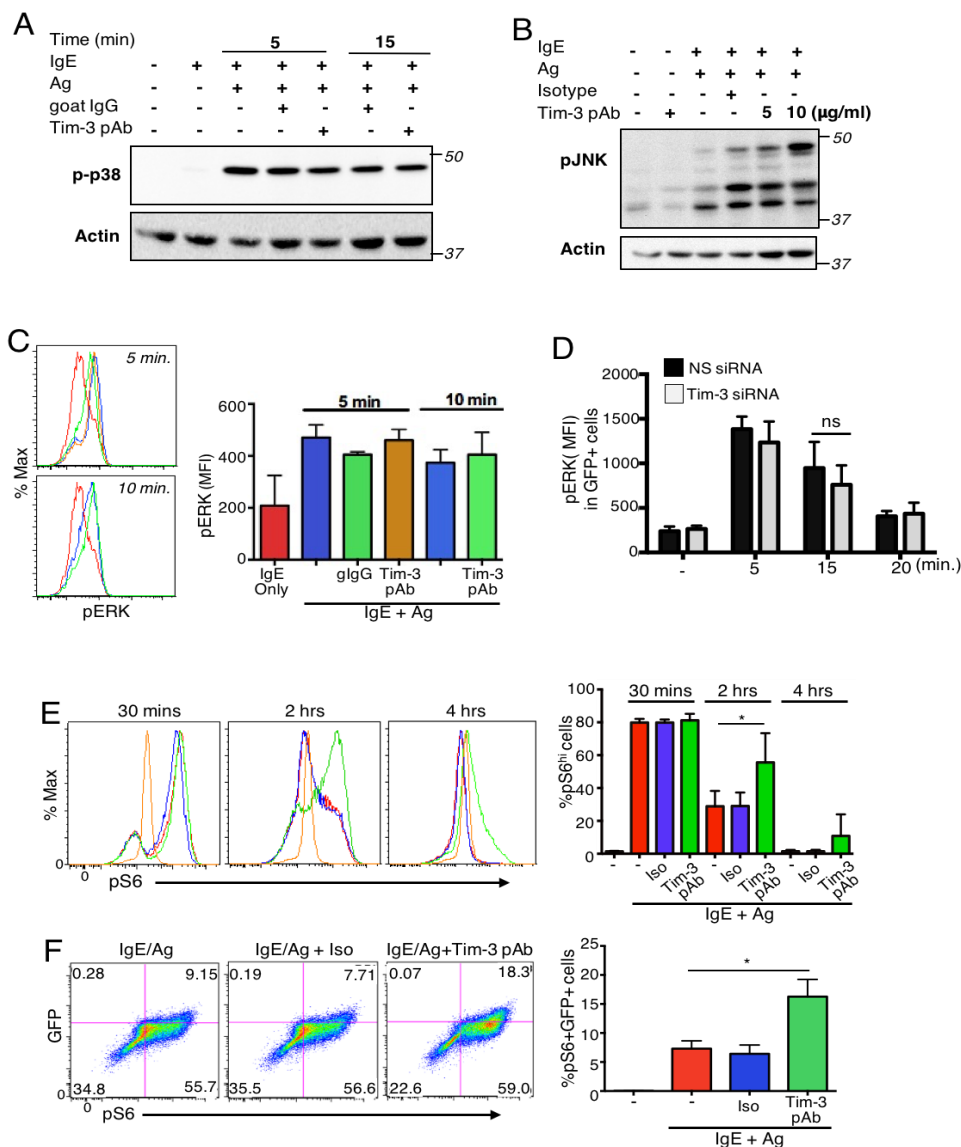


Figure 9-2: Tim-3 crosslinking enhances ribosomal protein S6 phosphorylation without affecting MAPKs signaling. BMMCs were stimulated as indicated, lysed and analyzed by western blotting for phospho-p38 (A), pJNK (B), and actin. Stimulated BMMCs were analyzed by flow cytometry for phospho-ERK (C). BMMCs were transfected with non-specific (NS) or Tim-3 siRNA and pmax-GFP for 48 hours. Transfected cells were stimulated as indicated and GFP⁺ cells were analyzed for phospho-ERK by flow cytometry (D). Nur77^{GFP} BMMCs were stimulated as indicated and analyzed for pS6 (S235/236) by flow cytometry. Results are quantitated as %pS6^{hi} (E) and %pS6+GFP⁺ cells (F). Results are representative of three independent experiments (A-B), two independent experiments (C-D), and average of three independent experiments (E-F). * p < 0.05.

9.4 DISCUSSION

We examined the potential signaling pathways at the Ag receptor proximal level and downstream that lead to enhanced degranulation and cytokine production by Tim-3 pAb and Ag co-treatment. Consistent with a co-stimulatory role, Tim-3 pAb alone could not activate mast cells. However, Tim-3 cross-linking led to increased Ag-mediated total tyrosine phosphorylation, specifically PLC- γ 1 phosphorylation, which is necessary for mast cell degranulation, yet failed to enhance Ag-activated phosphorylation of ERK, JNK or p38. PLC γ 1 is a substrate of Syk and increased PLC γ 1 activity implies that Syk activity may also be positively regulated by Tim-3 engagement. However, we did not observe increased Syk phosphorylation after Tim-3 co-stimulation. Syk is phosphorylated on a number of tyrosines, which are differentially regulated by either auto-phosphorylation or trans-phosphorylation by SFKs such as Lyn (131). Since we only examined phosphorylation of Y519 and Y520, indicators of Syk auto-phosphorylation, it is possible that Lyn-mediated phosphorylation of other activating tyrosines could be promoted by Tim-3 cross-linking. Phosphorylated Syk tyrosines can mediate either positive or negative regulatory Fc ϵ RI signaling (134-136). Therefore, it will be important to determine if the activation state of the inhibitory tyrosines, particularly Y317 associated with negative regulation of PLC γ 1 phosphorylation, is modified by Tim-3 activity.

Consistent with a co-stimulatory role, Tim-3 pAb alone could not activate mast cells. PLC γ 2 is also phosphorylated upon Fc ϵ RI stimulation, and is thus another potential Tim-3 target for enhanced NF-AT activation. In addition, the Tec kinase Btk may contribute to degranulation and cytokine production by phosphorylating PLC γ 1 and PLC γ 2 (143-145). Thus, future studies will also examine whether Btk recruitment to the plasma membrane or its activity can be altered

by Tim-3 engagement. Finally, calcium release, a critical functional read-out for increased PLC γ activation, warrants further investigation to support the connection between Tim-3 signaling and NF-AT activation.

PI3K/mTOR signaling pathways are crucial to mast cell survival, proliferation, protein synthesis and metabolism. Specifically, Fc ϵ RI-induced mTORC1, an upstream activator of S6, is constitutively active in human tumor mast cells (361). Here we showed that Tim-3 enhancement of Ag-activated cytokine secretion correlates with sustained S6 phosphorylation, suggesting a connection between Tim-3 signaling and the PI3K/mTOR pathway. Since the enhanced S6 phosphorylation was observed in conjunction with increased Nur77 expression, these results illustrated that modification of S6 activity is due to positive regulation of Ag receptor signal strength by Tim-3. Tim-3 pAb co-stimulation was previously shown to rescue IL-3 withdrawal-mediated apoptosis of mast cells, through induction of Ag-mediated IL-3 release (240). Overall, our data provide additional evidence to support a direct role for Tim-3 in mast cell survival, proliferation and cytokine production.

10.0 TIM-3 REGULATES POSITIVE VERSUS NEGATIVE ANTIGEN RECEPTOR SIGNALING

10.1 INTRODUCTION

Our *in vitro* studies thus far show that Tim-3 signaling can positively regulate IgE/Ag-mediated mast cell degranulation and cytokine production. Since mast cells are well-known effectors of type I hypersensitivity, we next explored whether a similar function for Tim-3 could be observed in an *in vivo* model of mast cell-dependent anaphylaxis, with the commonly used passive cutaneous anaphylaxis (PCA) model. During the immediate phase, mast cells-secrete histamine increases vascular permeability and edema, which can be measured by ear swelling. In the late-phase PCA, mast cell-dependent TNF secretion recruits polymorphonuclear cells (PMNs) into the site of inflammation ((362). Thus, we determine whether Tim-3 has a role in regulating PCA response in mast cells by comparing ear thickness, cytokine secretion, and infiltration of leukocytes between Tim-3 WT and KO mice. Unexpectedly, we observed heightened acute phase reaction in the absence of Tim-3 while cytokine production was not affected compared to WT littermates. As these results are reminiscent of those observed in the Lyn mutant *WeeB* mouse strain with reduced Lyn kinase activity (340), we assess how Tim-3 engagement might modulate mast cell activation in the acute absence of Lyn expression. Overall, our data reveal that Tim-3 enhances IgE/Ag-induced mast cell activation by regulating the positive and negative kinase activity of Lyn.

10.2 MATERIALS AND METHODS

10.2.1 Mice

Tim-3 KO mice were originally obtained from John Colgan (University of Iowa). Age-matched female wild-type C57BL/6 mice (7-8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed for seven days before use as control for PCA experiments. All studies were performed in accordance with University of Pittsburgh Institutional Animal Care and Use Committee procedures.

10.2.2 Antibodies and reagents

Monoclonal anti-dinitrophenyl (DNP) antibody, IgE isotype, clone SPE-7, DNP₃₂–HSA were from Sigma-Aldrich (St. Louis, MO). DNP₅–BSA was purchased from Biosearch Technologies (Petaluma, CA). Purified polyclonal antibody (Tim-3 pAb) and normal goat IgG control were from R&D Systems (Minneapolis, MN). Total Lyn antibody were from Cell Signaling Technology (Danvers, MA).

10.2.3 Passive cutaneous anaphylaxis (PCA)

Mice were sensitized by intradermal injection of anti-DNP IgE (50 ng/50 µl) or equal volume of vehicle (PBS) in each ear. Twenty-four hours later, mice were challenged i.v. with DNP₃₂-HSA (100 µg/200 µl in PBS). Mouse ear edema was evaluated by measuring ear swelling at indicated time points. The percent change in ear thickness of PBS- or IgE-treated ears was determined as

changes above baseline prior to antigen challenge. Mice were euthanized twenty-four post antigen challenge. Ear tissues were excised and digested using T-PER tissue extraction buffer (Thermo Fisher). Lysates were quantified for total protein amount and subsequent IL-6 measurement by ELISA (BioLegend). For histologic analysis, ear tissue sections were fixed in 4% PFA, and processed for hematoxylin and eosin. Tissue sections were examined with the AxioStar plus microscope equipped with epifluorescent and a digital camera (AxioCam; Zeiss, Oberkochen, Germany). Images were visualized with AxioVision (Zeiss). Results are represented as the mean \pm SEM. Two-way ANOVA analysis was performed with Bonferroni post hoc multiple comparison test. Outliers were removed using Outlier calculator (GraphPad software).

10.2.4 BMMC isolation and culture

Bone marrow cells from C57BL/6 WT mice were cultured in RPMI 1640 supplemented with 10% BGS, non-essential amino acid, 2-ME, HEPES, pen/strep-glutamine, and 20% IL-3-conditioned media for 4-6 weeks, after which >90% of viable cells are mature mast cells (c-kit⁺ Fc ϵ RI⁺ by flow cytometry).

10.2.5 BMMC stimulation and cytokine measurement

BMMCs were sensitized with 1 μ g/ml IgE overnight in complete media without IL-3. Cells were then stimulated with either DNP₃₂-HSA or DNP₅-BSA in IL-3-free media for indicated times. Supernatants were assayed for murine IL-6 by ELISA (BioLegend) six hours post-stimulation.

10.2.6 siRNA knock-down of Lyn in BMMC by nucleofection

BMMCs (3×10^6) were transfected with 100 pmol of non-specific or Lyn siRNA (Dharmacon) using the mouse macrophage nucleofector kit (Lonza), Y-001 program, and the Nucleofector II/2b device (Lonza). Transfected cells were collected after forty-eight hours and efficiency of Lyn knockdown was determined by western blotting.

10.2.7 Western blotting

Cells were lysed in 1% Nonidet-P-40 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) supplemented with AEBSF, aprotinin, leupeptin, pepstatin, sodium fluoride, sodium orthovanadate, and beta-glycerophosphate on ice for twenty minutes. Lysates were centrifuged at 4°C for fifteen minutes. For western blotting analysis, SDS-containing sample buffer was added to lysates prior to loading onto 10% SDS-PAGE gels for analysis of Lyn expression.

10.2.8 Statistical analysis

All statistical analysis was performed using Prism software (GraphPad Software). Paired, two-tailed Student's t-test, one-way and two-way ANOVA with Bonferroni post hoc test were used for data analysis and determination of p values, as appropriate.

10.3 RESULTS

10.3.1 Tim-3 functions through Lyn kinase to regulate FcεRI activating versus inhibitory signals

We hypothesized that the late-phase PCA response would be more impaired in the absence of Tim-3 compared to WT controls based on our *in vitro* findings. To our surprise, Tim-3 KO mice displayed a heightened immediate-phase PCA response compared to WT littermates, as measured by increased ear thickness (Fig. 10-1A). Since the peak of the immediate PCA response occurs around two hours post Ag injection and slowly declined, we were not able to observe any significant edema in the ear tissue twenty-four hours post Ag treatment (Fig. 10-1A-B). There appeared to be higher swelling in the vehicle (PBS) ear compared to the IgE treatment. This result is represented in the slight increase in ear thickness, suggesting this is an IgE-independent event (Fig. 10-1B). Similarly, cytokine production was comparable between WT and Tim-3 KO mice. However, the lack of IL-6 increase in IgE-treated ears over vehicle control suggests this may be a technical issue that needs to be addressed further (Fig. 10-1C).

We detected higher levels of serum IgE in some, but not all, naïve Tim-3 KO mice (Figure 10-1D), consistent with the slightly higher swelling observed in PBS-treated ears of Tim-3 KO mice (Fig. 10-1A). However, this would not seem to explain the higher PCA responses, since previous studies indicated that increased basal levels of IgE should result in higher receptor occupancy at baseline and less efficient priming with the exogenously administered IgE used in the PCA model (119),(340)). Nevertheless, these results indicate that the Tim-3 KO mice have a wider range of circulating serum IgE, which may be a confounding factor in our PCA experiments.

The PCA response of Tim-3 KO mice is reminiscent of enhanced anaphylaxis observed in the *WeeB* mouse strain, which expresses a mutant form of Lyn with reduced catalytic activity (340). Studies using *WeeB* mice and mast cells revealed that the majority of Lyn kinase activity is required for negative regulation of mast cell signaling, while only a small fraction of Lyn activity is needed to induce mast cell activation (340). Given the stronger anaphylactic response in the absence of Tim-3, it seemed possible that Tim-3 might be involved in tuning receptor signal strength through Lyn kinase, and subsequently its negative effects, leading to enhanced mast cell responses *in vivo*. To further investigate a possible intimate role for Tim-3 in regulating the relative activity of Lyn, we performed siRNA-mediated knockdown of Lyn in WT BMMCs. We then activated these cells with high or low valency antigen to engage its positive and negative functions, respectively, in the presence of Tim-3 crosslinking. Thus, under conditions of “weaker” stimulation, where Lyn primarily acts to enhance FcεRI signaling, knocking down Lyn expression resulted in decreased cytokine release that could be enhanced by Tim-3 pAb co-stimulation (Fig. 10-1E). Strikingly, under conditions of high intensity stimulation, where the negatively regulatory role of Lyn is revealed, Tim-3 cross-linking was still able to maintain, or even enhance, cytokine secretion (Fig. 10-F). The degree of Lyn knock-down (approximately 60%) is shown in Figure 10-1G. It should be noted that even with *in vitro* experiments using BMMC, we occasionally observed enhanced cytokine production by Tim-3 deficient mast cells, compared with WT mast cells, when stimulated with very high concentrations of antigen (data not shown).

Taken together, our results suggest an intimate role for Tim-3 in regulating the intensity of FcεRI signaling delivered through Lyn. Furthermore, the effects of Lyn knock-down allowed us to clarify the apparently paradoxical effects of Tim-3 knockout in the PCA model, where it is more difficult to control the strength of antigen receptor stimulation.

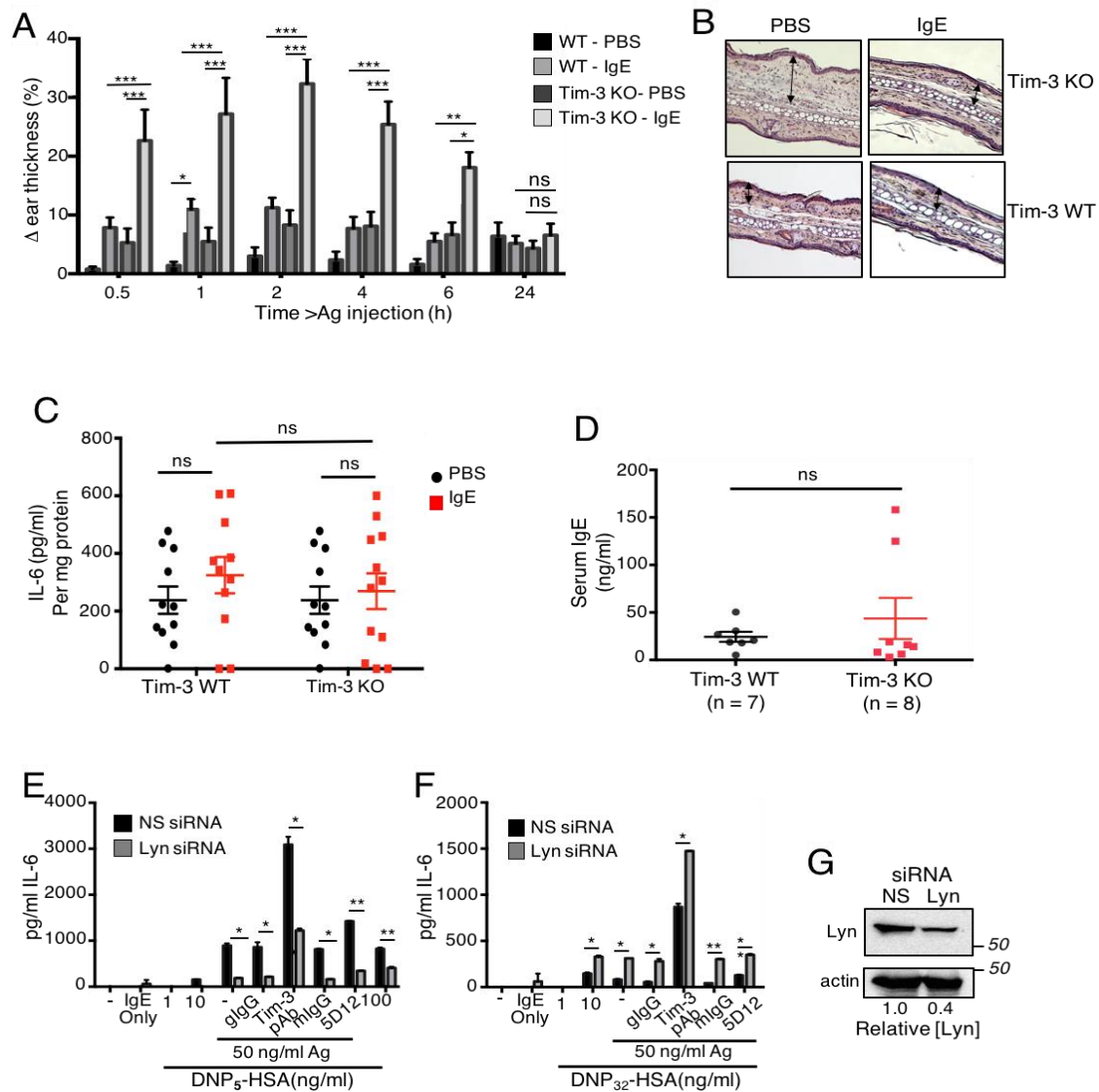


Figure 10-1: Tim-3 regulates Fc ϵ RI positive vs. negative signaling. PCA responses in WT (n=13) and Tim-3 KO (n=11) mice were measured as described in the *Materials and Methods*. Results are representative of three independent experiments totaling 19 WT and 14 Tim-3 KO mice. Results are represented as the mean \pm SEM. Two-way ANOVA analysis was performed with Bonferroni post hoc multiple comparison test. Outliers were removed using QuickCalcs (GraphPad Software). (D) Circulating serum IgE from Tim-3 WT and KO mice. Lyn siRNA-treated cells were sensitized with IgE and stimulated with low valency (E) or high valency (F) antigen, plus isotype control, Tim-3 pAb or Tim-3 mAb. (G) Efficiency of siRNA-mediated Lyn knockdown in BMMCs. Results shown are representative of three independent experiments performed in duplicate. *p < 0.05; **p < 0.005; ***p < 0.005.

10.4 DISCUSSION

Our PCA results revealed an unexpected Tim-3 involvement in balancing the positive and negative Ag receptor signaling. While we did not observe any difference in cytokine production, significant increase in ear thickness, an indication of tissue edema, was consistently observed in multiple experiments. In addition, we were not able to detect any TNF- α from the tissue even though it was one of the main cytokines driving cellular infiltration into the inflamed ears (data not shown and (362)). Therefore, more studies are needed to ensure reliable cytokine measurement to confirm the effects of Tim-3 on late-phase PCA response. An alternative is to perform immunofluorescent staining of IL-6 and TNF- α *in situ* to eliminate any potential technical issue with tissue extraction. It will be important to perform engraftment of WT, Tim-3 KO, or *WeeB* BMDCs into the mast cell-deficient c-kit^{W-sh/W-sh} mice to confirm the modulatory activity of Tim-3 on Ag receptor signaling. Alternatively, we can attempt to address the requirement for Tim-3 in mast cell-mediated allergic inflammation using the mast cell-dependent chronic lung inflammation model. In this model, mast cells are required for secretion of TNF to induce a Th2-biased response and lymphocyte recruitment into the airways and lungs (240).

We propose a model in which Tim-3 crosslinking provides a co-stimulatory signal (or set of signals) that integrates into the stimulatory signals that regulate IgE/Ag-mediated mast cell activation. In the absence of Tim-3, there is reduced functional output as a result of decrease Ag receptor intensity. However, Ag receptor signaling at high concentrations (or valency) of Ag is more complex, and the altered output may be attributed to reduced Lyn activity, most of which is involved in negative feedback signaling (340), leaving the residual kinase activity to enhance biological functions (Fig. 10-2). Performing the PCA assays with “weaker” antigen stimulation

(i.e. varying either the dose or valency) may help to confirm or refine this model. However, with the standard PCA model, the response rapidly declines to undetectable levels below the amount of antigen that we and others have used (340). Further testing of this model *in vivo* will require more sensitive assays for mast cell function, as well as more sophisticated models of conditional Tim-3 deficiency.

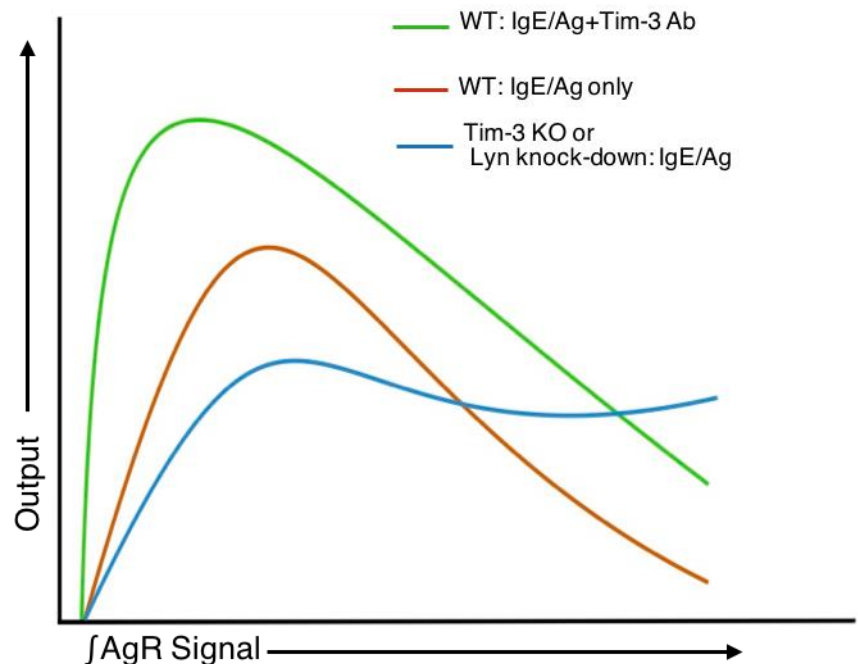


Figure 10-2: Proposed model for Tim-3 function on FcεRI signaling. Proposed model for the effects of Tim-3 engagement or deficiency on the functional outputs downstream of FcεRI signaling. Note that the effects of Tim-3 deficiency closely mirror those of partial loss-of-function of Lyn kinase activity.

11.0 TIM-1: TIM-4 INTERACTION ENHANCES CYTOKINE PRODUCTION WITHOUT AFFECTING DEGRANULATION

11.1 INTRODUCTION

Tim-4, mainly expressed on macrophages and dendritic cells, was identified as a ligand for Tim-1, and has been shown to co-stimulate T cell proliferation mediated by CD3/CD28 *in vitro*. Treatment with high avidity antibody to Tim-1 exacerbated severity of EAE in susceptible mice and broke EAE tolerance in a disease-resistant strain (241). However, Tim-4 can deliver an inhibitory or activating signal to T cells stimulated with low and high dose of CD3 respectively *in vitro* (195). Mast cells constitutively express surface Tim-1 and Tim-3, but not Tim-2 or Tim-4 (240). Tim-1 cross-linking by Tim4-Fc was shown to enhance cytokine production in a dose-dependent manner without affecting degranulation (240). Using a Tim4-Fc fusion protein, we determined if Tim-4 could augment FcεRI-mediated mast cell activation under both high and low Ag valency stimulation that has been known to engage FcεRI negative and positive signaling pathways respectively (109). Here, we show that Tim4-Fc promotes Ag-induced mast cell cytokine production without affecting degranulation. In addition, Tim4-Fc is more efficient at enhancing cytokine release at high Ag valency, thus suggesting a role for Tim-1 signaling in preventing engagement of Ag negative signaling.

11.2 MATERIALS AND METHODS

11.2.1 BMMC isolation and culture

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) for bone marrow isolation. Bone marrow cells were maintained in RPMI 1640 supplemented with 10% BGS, non-essential amino acid, 2-ME, HEPES, pen/strep-glutamine, and 20% IL-3-conditioned media for 4-6 weeks, after which >90% of viable cells are mature mast cells (c-kit⁺ FcεRI⁺ by flow cytometry).

11.2.2 Antibodies and reagents

Monoclonal anti-dinitrophenyl (DNP), IgE isotype, clone SPE-7, DNP₃₂-HSA, and 4-Nitrophenyl N-acetyl-β-D-glucosaminide (pNAG) were purchased from Sigma-Aldrich (St. Louis, MO). DNP₅-BSA was from Biosearch Technologies (Petaluma, CA). Purified Tim4-Fc were obtained from Vijay Kuchroo (Harvard Medical School). Human IgG as isotype control for Tim4-Fc was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-mouse Fc block (2.4G2) was purchased from BD Biosciences (San Jose, CA).

11.2.3 BMMC stimulation and cytokine measurement

BMMCs were sensitized overnight with 1 μg/ml IgE without IL-3 conditioned media. Cells were stimulated with DNP₃₂-HSA or DNP₅-BSA, with either human IgG isotype or Tim4-Fc for indicated times. Six hours post stimulation, supernatants were collected and assayed for murine IL-6 and TNF-α by ELISA (BioLegend).

11.2.4 Measurement of beta-hexosaminidase release

BMMCs were sensitized and subsequently stimulated for thirty minutes in Tyrode's buffer (135 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1.8 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, and 0.5 mg/ml BSA). Supernatants (stimulated release) were collected and cells were lysed (content) with 0.5 % Triton-X100 in PBS for fifteen minutes on ice. Content and stimulated release fractions were incubated with 1mM pNAG substrate for 1 hour at 37⁰C. Carbonate buffer (0.1M, pH 9.0) was added to stop reaction and absorbance was obtained at 405nm on a plate reader. Percentage of beta-hexosaminidase release was calculated as $(\text{release}^{\text{stimulated}}/\text{content}^{\text{total}}) \times 100$.

11.2.5 Mast cell degranulation by Annexin V-based flow cytometry

Degranulation was measured as described previously (321, 363). Briefly, BMMCs were loaded with 0.1 μ M of LysoTracker Deep Red (Invitrogen) for thirty minutes prior to sensitization with 0.5 μ g/ml IgE for 1 hour at 37⁰C. Ninety minutes post antigen cross-linking by DNP₃₂-HSA, cells were collected and stained with Annexin V (BioLegend). %degranulation was determined as percentage of BMMCs that is AnnexinV⁺LysoTracker^{lo}.

11.2.6 Statistical analysis

All statistical analysis was performed using Prism software (GraphPad Software). Paired, two-tailed Student's t-test, one-way and two-way ANOVA with Bonferroni post hoc test were used for data analysis and determination of p values, as appropriate.

11.3 RESULTS

11.3.1 Tim-1 engagement by Tim4-Fc enhances IgE/Ag- stimulated mast cell cytokine production without affecting degranulation

Similar to published findings (96), we observed similar effects of Tim4-mediated IL-6 production in IgE/Ag-stimulated BMMCs (Fig. 11-1B). In addition, we quantified the degranulation response by measuring beta-hexosaminidase release as well as with a flow cytometry-based assay that tracks both Annexin V binding to exposed phosphatidylserine (PS) and loss of LysoTracker staining, due to granule release (AnnexinV+LysoTracker^{lo}). The latter method has the advantage of a high signal-to-noise ratio, and as such has been a robust assay for evaluating IgE/Ag-stimulated mast cell degranulation (321, 363). Thus, we showed that varying the concentration of Tim-4, along with suboptimal antigen concentration to observe potential co-stimulation, did not have an effect on the immediate degranulation response (Fig. 11-1C-D). These results indicate that Tim-1 ligation by Tim-4 can exert differential effects on the immediate degranulation response, vs. late-phase cytokine production.

Antigen valency and concentration have been shown to control the outcomes of FcεRI engagement in not just quantitative, but also qualitative, fashions (Xiao, 2005). Specifically, low antigen concentration or valency will activate only positive FcεRI signaling pathways, while high antigen valency or concentration will preferentially engage negative signaling components downstream of FcεRI. This is due to activity of the Src family kinase Lyn as a positive and/or negative regulator of FcεRI signaling at low or high antigen valency, respectively (109). We stimulated BMMCs with low (DNP₅-BSA) or high (DNP₃₂-HSA) potency antigens in the presence

of Tim4-Fc or isotype control and assessed whether Tim-1 ligation could contribute to receptor signaling intensity. Thus, at high antigen valency, which also activates negative feedback of antigen receptor signaling, Tim-4 was able to maintain high IL-6 production but not at low antigen valency that induces robust antigen receptor signaling (Fig. 11-1E-F). Specifically, increasing the amount of Tim-4 further promoted cytokine secretion under high valency antigen stimulation, i.e. under conditions where the negative signaling loop is triggered. To exclude the possibility that Fc γ receptor binding may interfere with Tim1-Tim4 interaction, we compared IL-6 release by BMMCs, with or without addition of Fc blocking antibody, and found no differences, at least at the concentration of Tim4-Fc used throughout this study (5 μ g/ml) (Fig. 11-1E-F). These findings indicate that Tim-1 ligation can modulate the intensity of the antigen-induced positive Fc ϵ RI signaling pathways and may be able to bypass the negative feedback signaling loop controlled by Lyn.

To determine if Tim-4 treatment could affect production of other mast cell mediators, we compared supernatant from cells co-stimulated with Ag and Tim4-Fc to cells stimulated with Ag alone in a chemiluminescence-based mouse cytokine array. Thus, Ag alone induced production of well-known mast cell cytokines and chemokines including IL-6, TNF α , IL-13, CCL2, and CCL3, all of which were not detectable in the Tim4-Fc-treated sample. Furthermore, Tim-1 cross-linking promoted secretion of IL-7 and IL-12p70 from Ag-activated cells (Fig. 11-2). Therefore, it remains to be seen whether Tim-1 ligation engages specific signaling pathways for production of a distinct set of cytokines or augments the overall activation state of the mast cells.

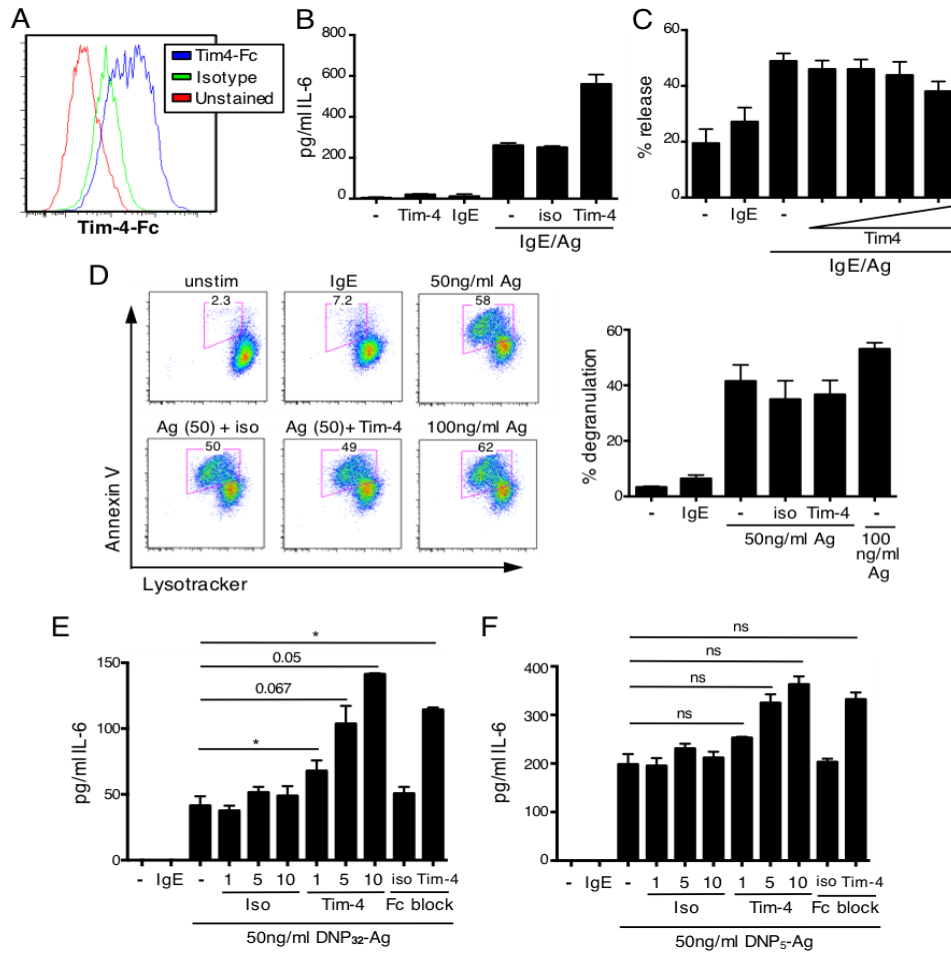


Figure 11-1: Tim4-Fc enhances IgE/Ag-mediated mast cell cytokine production at high but not low antigen valency stimulation without affecting degranulation. BMMCs were incubated with 0.5 μ g of Tim4-Fc or human IgG (iso) on ice for 20 minutes followed by flow cytometry analysis (A). BMMCs were sensitized overnight with 1 μ g/ml of IgE and stimulated with 50 ng/ml of DNP₃₂-HSA, alone or with 5 μ g/ml of Tim4-Fc or “iso” for six hours prior to IL-6 measurement by ELISA (B). BMMCs were stimulated as indicated in the presence of increasing amount of Tim4-Fc (1-50 μ g/ml) for thirty minutes prior to degranulation measurement by means of beta-hexosaminidase release (C). Cells were loaded with Lysotracker Deep Red, sensitized with IgE, and stimulated with antigen plus isotype control or Tim4-Fc for ninety minutes prior to Annexin V staining and flow cytometry analysis (D). IgE-sensitized BMMCs were pre-treated with anti-mouse Fc blocking Ab (2.4G2) for ten minutes prior to stimulation with DNP₃₂-HSA (E) or DNP₅-HSA (F) alone or in conjunction with indicated amount of control Fc (“iso”) or Tim4-Fc for six hours. IL-6 cytokine production was determined by ELISA. Results are representative of three independent experiments with duplicates in each. * p <0.05.

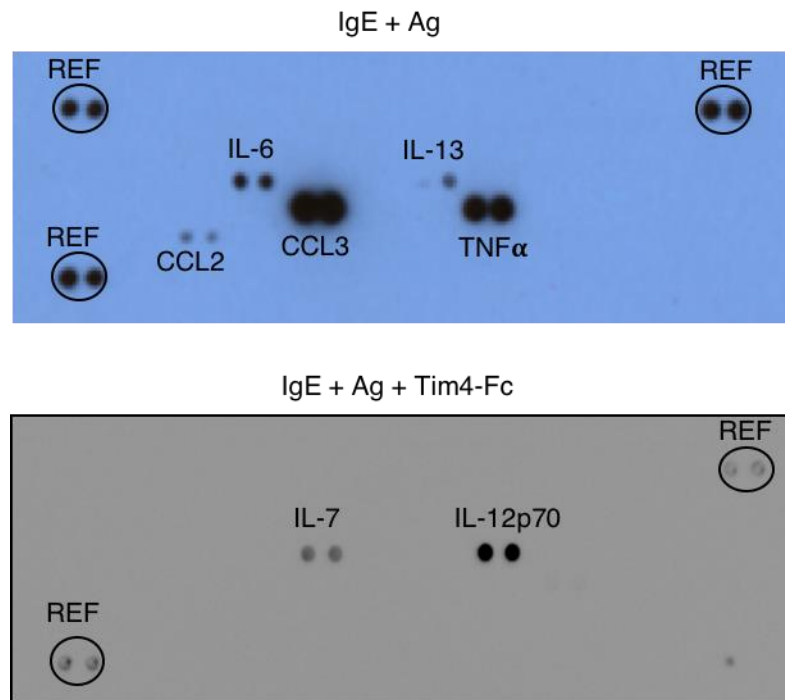


Figure 11-2: Ag and Tim4-Fc co-stimulate release of distinct cytokines compared to Ag alone. BMMCs were sensitized with IgE prior to stimulation with 50 ng/ml Ag, with or without 5 μ g/ml Tim4-Fc for six hours. Supernatant was collected and subjected to mouse cytokine array detection.

11.4 DISCUSSION

Tim-4 treatment consistently enhanced IgE/Ag-mediated cytokine production but not degranulation, which may be a time- and/or signal intensity-dependent effect. Tim-4 was reported to have bimodal effects, either enhancing or inhibiting T cell proliferation, depending on anti-CD3/CD28 concentrations (195). This bimodal regulation was later reported to be inhibitory for naïve T cells, which do not express Tim-1, and co-stimulatory for activated T cells, suggesting that Tim-4 either binds to an unknown ligand expressed only on naïve T cells or that Tim-4 has a higher affinity for Tim-1 expressed on activated T cells (364). In addition, Tim-4 can bind to naïve T cells that do not express Tim-1 and inhibit Th17 differentiation (365). This effect was shown to be independent of Tim-1 activity, since addition of Tim-1 blocking antibody, presumably to block the Tim1:Tim4 interaction, could not rescue Tim4-mediated inhibition (365). In contrast to such ligand-dependent effects observed on T cells, Tim-4 co-stimulates IgE/Ag-mediated mast cell activation by cross-linking Tim-1. Using low and high valency antigens to engage the positive and negative signaling pathways of FcεRI, respectively, we showed that Tim-4 could enhance mast cell cytokine production in both settings. Specifically, Tim-4 co-stimulated cytokine release in a dose-dependent manner, under both high and low valency antigen stimulation, albeit less efficiently with low valency Ag. These results imply that Tim-4 contributes to FcεRI signaling intensity and/or duration, and may potentially override negative feedback signals linked primarily to Lyn-mediated phosphatase activation. Together with findings that Tim-4 alone does not induce cytokine production in mast cells, our results demonstrate that Tim-1 signaling interfaces with common effector molecules downstream of FcεRI signaling, rather than acting through a parallel pathway, to enhance mast cell functions.

12.0 TIM-1 ANTIBODIES DO NOT MODULATE MAST CELL ACTIVATION

12.1 INTRODUCTION

Since Tim-1 ligation with Tim-4 exhibited both positive and negative effects on T cell responses, it raised the possibility that Tim-4 may either have a different ligand on T cells or function as a dual regulator on T cells (195). These results also imply that Tim-1 may be a positive or negative regulator of T cell function depending on the molecule is engaged during T cell activation. Therefore, antibodies specific to Tim-1 have been generated to determine how antibody affinity and/or avidity can affect T cell activity. Of significance are the monoclonal antibodies (mAb) 3B3 and RMT1-10, which have been termed “agonistic” and “antagonistic” antibodies, respectively, due to their ability to enhance or inhibit effector T cell activation (221). Since then, several antibodies against Tim-1 have been tested and showed no effects on either IgE/Ag-induced mast cell degranulation or cytokine production (240). Here, we determine whether antibody-mediated Tim-1 cross-linking can modulate Ag-induced cytokine production in a dose-dependent manner. Our results show that the existing Tim-1 antibodies with known function effects on T cells and B cells do not regulate FcεRI-induced mast cell activation, suggesting they may have cell type-dependent effects (195, 220, 221, 232, 240, 241).

12.2 MATERIALS AND METHODS

12.2.1 BMMC isolation and culture

Bone marrow cells from C57BL/6 mice were cultured in RPMI 1640 supplemented with 10% BGS, non-essential amino acid, 2-ME, HEPES, pen/strep-glutamine, and 20% IL-3-conditioned media for 4-6 weeks, after which >90% of viable cells are mature mast cells (c-kit⁺ FcεRI⁺ by flow cytometry).

12.2.2 Antibodies and reagents

Monoclonal anti-dinitrophenyl (DNP), IgE isotype, clone SPE-7, and DNP₃₂-HSA, were purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies to murine Tim-1 (3B3, RMT1-10, 5G5, 5F12) were obtained from Vijay Kuchroo (Harvard Medical School). Monoclonal antibodies to murine Tim-1 (4A2, 5D1, 4G8, 4B2) were originally developed at Biogen and obtained under an MTA with CoStim Pharmaceuticals (Cambridge, MA). Purified rat IgG2a as isotype was from eBioscience (San Diego, CA).

12.2.3 BMMC stimulation and cytokine measurement

BMMCs were sensitized overnight with 1μg/ml IgE without IL-3 conditioned media. Cells were stimulated with DNP₃₂-HSA and either rat isotype or the indicated Tim-1 antibodies. Six or twenty-four hours post stimulation, supernatants were collected and assayed for murine IL-6 and TNF-α by ELISA (BioLegend)

12.3 RESULTS

12.3.1 Tim-1 antibodies do not exert any effects on Ag-induced mast cell cytokine release

We tested several antibodies that have been shown to modulate T cell responses including the well-characterized mAb's 3B3 and RMT1-10 (195, 221, 232, 240, 241). Compared to Tim4-Fc, we did not observe any dose-dependent effects of 3B3, RMT1-10, the mucin domain-specific mAb 5G5, and the IgV domain-binding mAb 5F12 on levels of IL-6 and TNF- α secreted by IgE-sensitized and Ag-stimulated BMMCs at six or twenty-four post stimulation (Fig. 12-1A-H). We examined several other antibodies, generated against the BALB/c allele of Tim-1, which were shown to either exacerbate or ameliorate Th2-dependent OVA-induced lung inflammation in mice (220). Specifically, the mAb's 3A2 and 4A2 bind to distinct epitopes near an N-linked glycosylation site in the stalk region and the IgV domain respectively and both can reduce lung inflammation and pathology (220). However, aside from 4G8, we detected neither significant binding of these antibodies to BALB/c Tim-1 nor any changes in cytokine production (Fig. 12-2A-C). Even though these antibodies were raised against the BALB/c allele of Tim-1, we also observed binding of 4G8 to C57BL/6 Tim-1 (Fig. 12-2D). There was a small, although statistically insignificant, increase in IL-6 production when 4G8 was used in co-stimulation with Fc ϵ RI crosslinking by IgE/Ag (Fig. 12-2E). These results further support the concept that the effects of antibody modulation of Tim-1 are cell-type and context-dependent (220, 240).

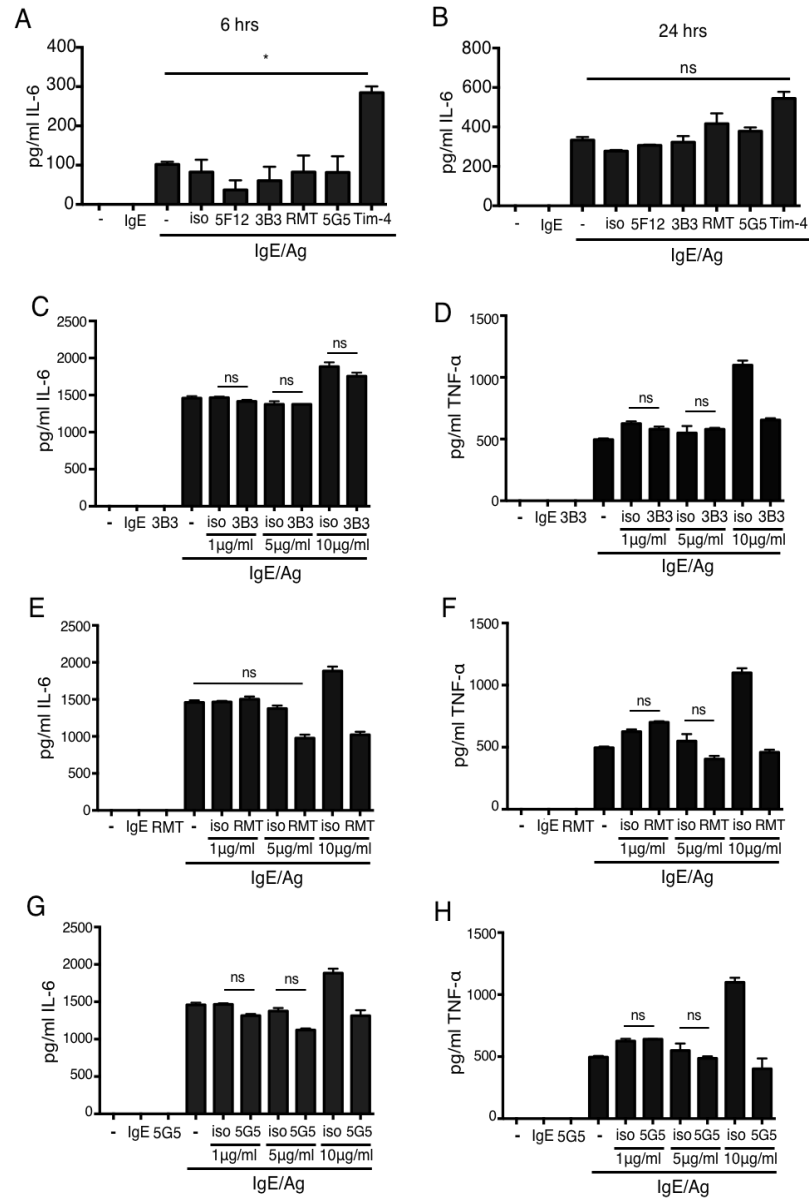


Figure 12-1: Tim-1 antibodies did not significantly alter IgE/Ag-mediated mast cell cytokine production compared to ligation by Tim4-Fc. C57BL/6 BMMCs were sensitized with IgE overnight and stimulated with DNP₃₂-HSA in the presence of isotype control or monoclonal antibodies against Tim-1 3B3, RMT1-10, 5F12, 5G5 or Tim4-Fc for six (A) or twenty-four (B). BMMCs were stimulated as indicated for six hours (C-H). Supernatant was collected for IL-6 and TNF-α analysis by ELISA. Results are representative of two (A-B) and at least three independent experiments performed in duplicates (C-H). *p<0.05.

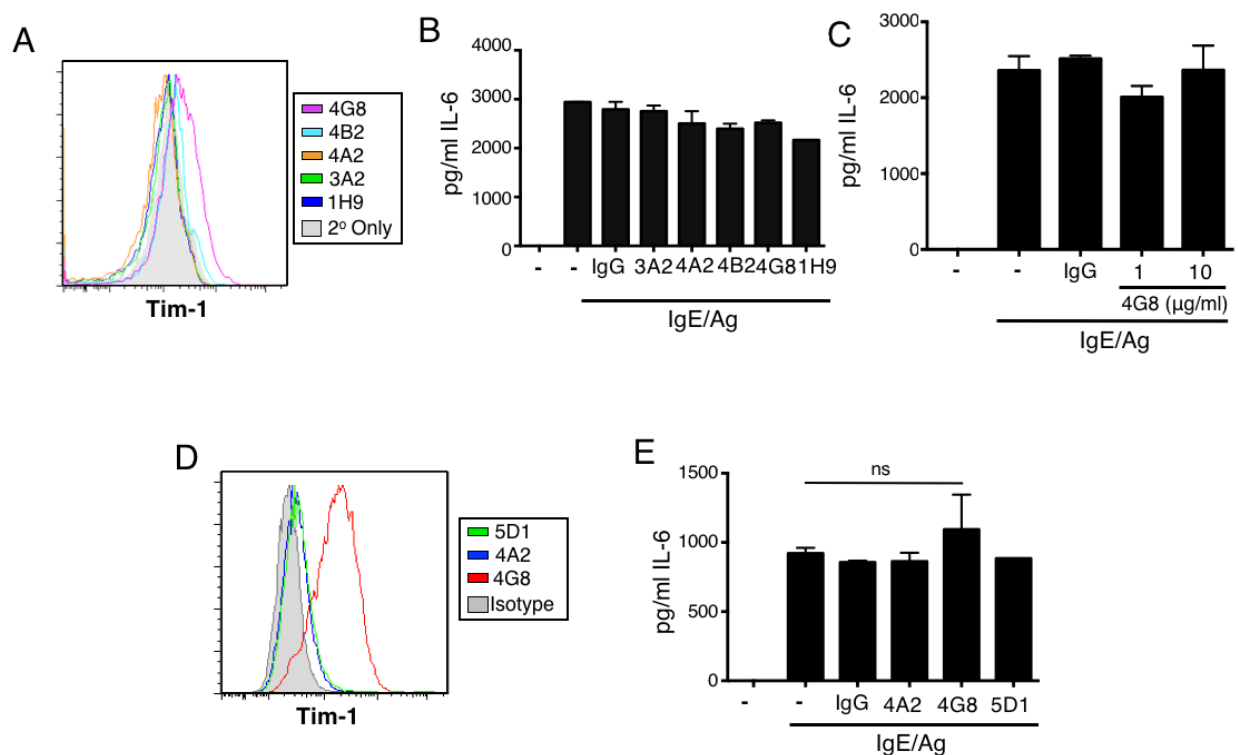


Figure 12-2: Specific antibodies to BALB/c Tim-1 do not modulate IgE/Ag-mediated mast cell cytokine production. The indicated antibodies were incubated with BALB/c BMMCs for 30 minutes on ice followed by anti-rat IgG-Alexa-647 secondary antibody, prior to flow cytometry analysis (A). BALB/c BMMCs were sensitized with IgE overnight and stimulated with DNP₃₂-HSA in the presence of isotype control or monoclonal antibodies 3A2, 4A2, 4B2, 4G8, 1H9 (B-C) for six hours. Culture supernatants were analyzed for IL-6 and TNF- α by ELISA. BL/6 BMMCs were incubated with 4G8, 4A2 and 5D1 antibodies followed by anti-rat IgG-Alexa 647 secondary antibody prior to flow cytometry analysis (D). BL/6 BMMCs were sensitized with IgE overnight and stimulated with DNP₃₂-HSA together with either isotype control or the indicated antibodies for six hours prior to IL-6 measurement by ELISA (E). Results are representative of three independent experiments performed in duplicates.

12.4 DISCUSSION

In this study, we aimed to determine how Tim-1 cross-linking by antibodies could modulate mast cell activation. Unlike in T cells or *in vivo* experiments, we and others found that mast cells did not respond to Tim-1 antibody treatment, as none of the antibodies elicited a change in mast cell degranulation or cytokine production (Fig. 11-1, 11-2, and (240)). Among the antibodies with defined epitopes and functional effects on T cells (220, 240), we observed very weak binding to Tim-1 by most of these antibodies, suggesting glycosylation patterns of Tim-1 among cell types may affect antibody recognition. The Tim-1 mucin and stalk region is heavily glycosylated with multiple N- and O—linked carbohydrate side chains (190). As a result, two different mAb's recognizing the Tim-1 stalk elicited opposing effects *in vivo*, suggesting that antibody-mediated Tim-1 targeting may affect Tim-1 interaction with its ligands or with itself (220). Since Tim-4 is not expressed on mast cells, and thus is not an interference in Tim-1 recognition by antibodies. Phosphatidylserine (PS), another Tim-1 ligand, is present in our mast cell culture and binds primarily to the IgV domain of Tim-1. However, PS is unlikely to compete with Tim-1 antibodies for binding since it requires calcium ion to facilitate interaction with Tim-1, a component that is absent from our staining buffer. Overall, our results showed that Tim-4 is effective in ligating Tim-1, leading to enhanced Ag-stimulated mast cell cytokine production without affecting degranulation while the existing Tim-1 antibodies largely have no functional effects on mast cell responses.

13.0 TIM-1 TRANSIENT EXPRESSION PROMOTES ANTIGEN-STIMULATED TRANSCRIPTIONAL ACTIVATION IN MAST CELLS

13.1 INTRODUCTION

We previously demonstrated that transient expression of Tim-1 co-stimulated TCR/CD28-mediated transcriptional activation of IL-4 and IFN- γ production and NF-AT/AP1-dependent transcription. This co-stimulatory activity was dependent on tyrosine 276 in the Tim-1 cytoplasmic tail (222). Furthermore, phosphorylation of tyrosine 276 occurred in an Lck-dependent manner and allowed for recruitment of the p85 α and β subunits of the PI3K, leading to activation of the downstream kinase Akt and subsequent activation of the transcription factors NFAT and AP-1 (242). While Tim-1 ligation enhances Fc ϵ RI-mediated mast cell activation, the precise signaling mechanisms by Tim-1 are currently unknown. Here, we show that that ectopic expression of Tim-1 alone can augment IgE/Ag-triggered NF- κ B and NFAT/AP-1 activation, leading to enhanced IL-6 transcriptional activation and cytokine production. Furthermore, our data provide evidence that these events are dependent on the tyrosine phosphorylation of Tim-1 cytoplasmic tail, further highlighting the importance of tyrosine phosphorylation in mediating Tim-1 co-stimulatory activity.

13.2 MATERIALS AND METHODS

13.2.1 Antibodies and reagents

Monoclonal anti-dinitrophenyl (DNP), IgE isotype, clone SPE-7, DNP₃₂-HSA, and anti-FLAG M2 antibody were purchased from Sigma-Aldrich (St. Louis, MO). IL-6 luciferase reporter constructs were obtained from Sarah Gaffen (University of Pittsburgh), originally from Oliver Eickelberg (Helmholtz Zentrum Munchen).

13.2.2 Transcriptional reporter assays

MC/9 mast cell line was maintained in DMEM supplemented with 10% BGS, 2-ME, Pen/Strep with Glutamine, and 10% IL3-conditioned media. MC/9 cells (15×10^6) were transfected with 15 μ g of IL6-luc, NF- κ B-luc, or NFAT/AP1-luc with 5 μ g of pCDEF3 (empty vector), FLAG-tagged Tim-1 full length (FL), Tim-1 cytoplasmic deletion (Δ cyto), or Tim-1 tyrosine mutant (Y276F). Electroporation was performed at 290V, 950 μ F using a Gene Pulser II apparatus (Bio-Rad). Transfected cells were collected twenty-four hours and stimulated with 0.5 μ g/ml IgE and indicated amount of DNP₃₂-HSA as antigen for six hours. Luciferase assays were performed as previously described (366).

13.2.3 Statistical analysis

All statistical analysis was performed using Prism software (GraphPad Software). Paired, two-tailed Student's t-test, one-way and two-way ANOVA with Bonferroni post hoc test were used for data analysis and determination of p values, as appropriate.

13.3 RESULTS

13.3.1 Tim-1 expression co-stimulates IgE/Ag-induced NF- κ B, NFAT/AP1, and IL-6 transcriptional activation in a tyrosine phosphorylation-dependent manner

Similar to our findings in T cells, ectopic expression of Tim-1 on MC/9 mast cells was able to enhance IgE/Ag-stimulated NF- κ B transcriptional activation (Fig. 13-1A). This enhancement was abrogated when the cytoplasmic tail of Tim-1 was deleted or when a tyrosine-phenylalanine mutant (Y276F) was used (Fig. 13-1A). Polymorphisms in Tim-1 have been associated with differential responses to OVA-induced allergic asthma (190). We found that both isoforms of Tim-1 (BL/6 and BALB/c) could significantly enhance activation of an NF- κ B transcriptional reporter to a comparable extent (Fig. 13-1B). Consistent with findings in T cells and effects on mast cell cytokine production, transient expression of Tim-1 also up-regulated NF-AT/AP1 promoter activation, in a tyrosine phosphorylation-dependent manner (Fig. 13-1C).

Since Tim4-Fc co-stimulated IgE/Ag-mediated mast cell IL-6 production, we determined if Tim-1 could promote IL-6 transcriptional activation using the previously described IL-6

promoter mutant constructs (363). Thus, expression of full length Tim-1 promoted IL-6 reporter activity, in a tyrosine phosphorylation-dependent manner (Fig. 13-2A). Using IL-6 promoter deletion constructs, we showed that Tim-1 mediated enhancement of transcriptional activation and subsequent production of IL-6 primarily through activation of NF- κ B and AP-1 transcription factors (Fig. 13-2B). While we have occasionally observed Tim-1-driven enhancement of IL-6 reporter activity when the C/EBP binding site is absent, the Tim-1 effects are mainly through activating NF- κ B and AP-1 since an IL-6 promoter mutant lacking both NF- κ B and C/EBP binding site could not rescue the inhibition of IL-6 transcriptional activity due to lack of NF- κ B activity (Fig. 13-2C-D). Unlike Tim-4 cross-linking of Tim-1, addition of anti-FLAG antibody could not further promote reporter activity, suggesting either that Tim-1 may have other ligands on MC/9 mast cells or that Tim-1 can homodimerize after ectopic expression, via its heavily glycosylated mucin domain, leading to downstream signaling (Fig. 13-2A).

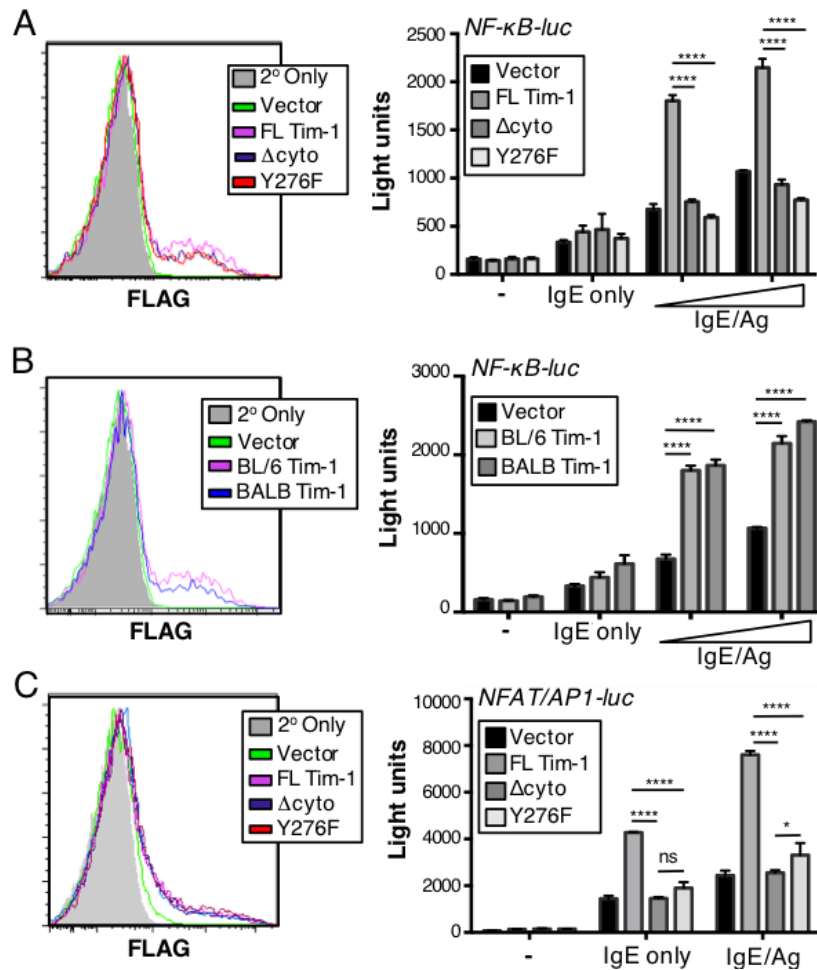


Figure 13-1: Tim1-mediated enhancement of NF-κB and NF-AT/AP1 transcriptional activation is dependent on a tyrosine on its cytoplasmic tail. MC/9 mouse mast cells were transfected with empty vector (pCDEF3), BL/6 or BALB/c Tim-1 (full length (FL)), BL/6 Tim-1 lacking the cytoplasmic region (Δcyto) or full length Tim-1 (BL/6) with tyrosine to phenylalanine mutated at tyrosine 276 (Y276). Transfected cells were stimulated with 0.5 μg/ml IgE plus either 30 ng/ml or 100 ng/ml DNP₃₂-HSA for six hours. MC/9 mast cells were co-transfected NF-κB (A-B), or NFAT/AP1 (C) luciferase reporters. Results are representative of three independent experiments performed in triplicate. *p<0.05, **p<0.005, ****p<0.00005.

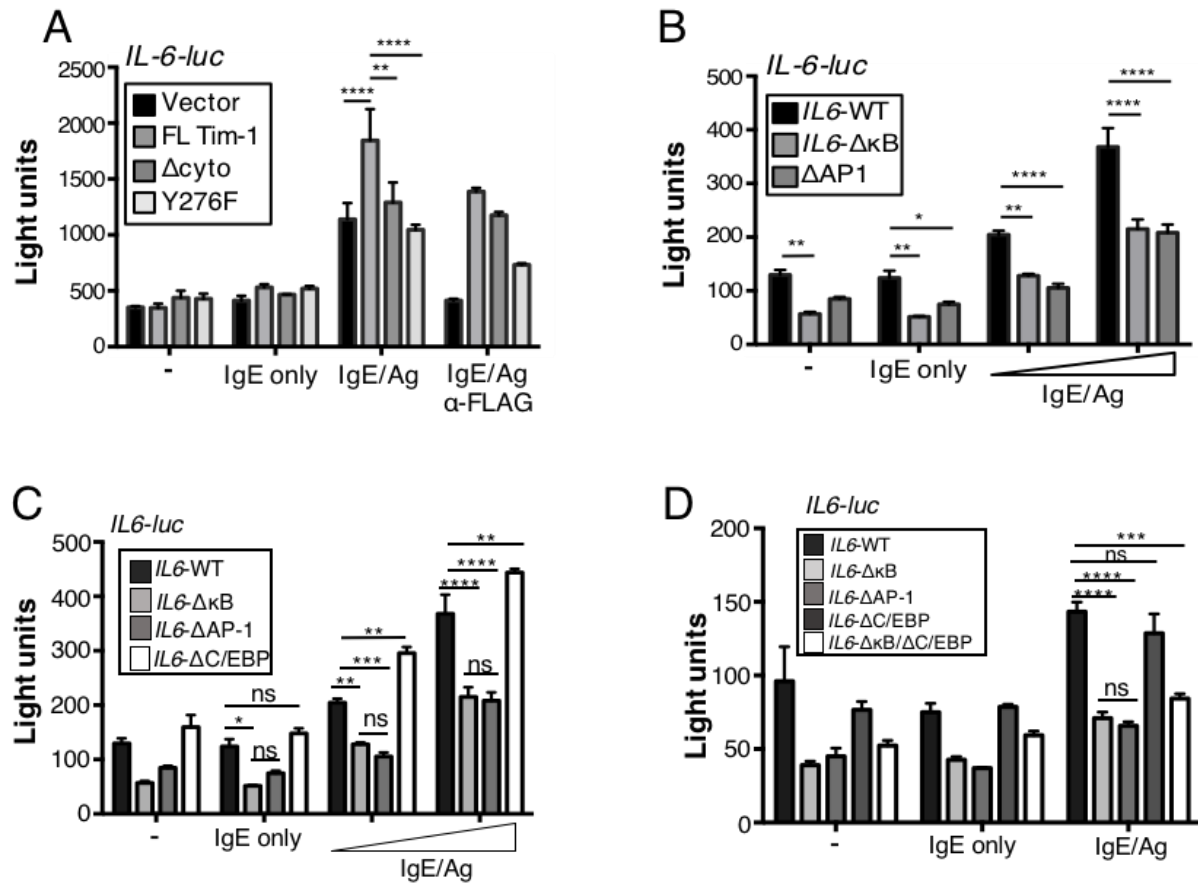


Figure 13-2: Tim-1 enhances IgE/Ag-mediated IL-6 transcriptional activation through NF-κB and AP1 activation in a tyrosine phosphorylation-dependent manner. MC/9 mast cells were transfected with full length BL/6 Tim-1 along with the indicated IL-6 luciferase reporters (A-D). Transfected cells were stimulated with 0.5 μg/ml IgE plus either 30 ng/ml or 100 ng/ml DNP₃₂-HSA, with or without addition of anti-FLAG antibody for six hours. Results are representative of three (A, B, D) and two (C) independent experiments performed in triplicate. *p<0.05, **p<0.005, ***p<0.0005; ****p<0.00005.

13.4 DISCUSSION

Mast cells constitutively express high levels of cell-surface Tim-1, a molecule with co-stimulatory effects on many immune cell types, but with unclear mechanisms of action. Here we demonstrate that Tim-1 is a positive regulator of mast cell activation and cytokine production. Similar to our findings on the effects of Tim-3 on mast cells (363), Tim-1 expression alone could promote IgE/Ag-mediated NF- κ B and NF-AT/AP1 transcriptional activation, without additional cross-linking antibodies or exogenous ligands. Tim-4 is a ligand for Tim-1, but the lack of Tim-4 expression on mast cells makes it an unlikely explanation for this particular role of Tim-1 in mast cells. Tim-1 can also bind PS on apoptotic cells (214) or PS transiently exposed on degranulating mast cells, either of which could potentially contribute to enhance Tim-1 signaling, although whether PS binding to Tim-1 can lead to mast cell activation is still unknown. Tim-1 has also been reported to bind LMIR5/CD300b, a DAP12-coupled activating receptor expressed on myeloid cells (367). Thus, stimulation with TIM1-Fc was able to induce LMIR5-mediated ERK activation in mast cells, suggesting that LMIR5 is another potential endogenous ligand of Tim-1, driving the enhancement of transcriptional response. Finally, Tim-1 may homodimerize through its heavily glycosylated mucin domain, leading to its phosphorylation and downstream function. Regardless of its mode of activation, we showed that Tim-1 co-stimulation is dependent on the tyrosine phosphorylation motif of Tim-1 cytoplasmic tail as mutation of tyrosine 276 rendered Tim-1 unable to mediate its co-stimulatory function. The Src kinase Fyn has been shown to phosphorylate Tim-1 in a B cell line (368). We showed that the Tim-1 cytoplasmic tail is phosphorylated upon TCR stimulation in an Lck-dependent manner and can recruit p85 binding (242). Therefore, Src family kinases like Lyn, Fyn or Hck are potential facilitators of Tim-1 phosphorylation upon IgE/Ag activation.

14.0 TIM-1 MUCIN DOMAIN IS NOT REQUIRED FOR TIM4-MEDIATED ENHANCEMENT OF MAST CELL CYTOKINE RELEASE

14.1 INTRODUCTION

Our findings thus far suggest that the Tim1-Tim4 interaction augments FcεRI signaling itself, rather than acting through a parallel pathway, since Tim-4 treatment alone does not induce any significant cytokine production or degranulation. While the Tim1-Tim4 interaction has been attributed primarily to the IgV domain, Tim-4 has also been proposed to bind to the Tim-1 mucin domain (195). Intriguingly, the genetic linkage of Tim-1 to allergies and asthma is associated with polymorphisms in the mucin domain. On the other hand, two separate strains of Tim-1-deficient mice showed relatively unaltered IgE production and AHR development in an OVA-induced mouse model of asthma, even though one study did observe higher type 2 and Th17 cytokine production in Tim-1 knockout (KO) mice (238, 239). Furthermore, the importance of the Tim-1 mucin domain has been demonstrated in T cell activation, differentiation, trafficking, and effector function in autoimmunity and airway inflammation (202, 365). It is also essential to regulatory B cell maintenance, signaling, transplant tolerance and induction of systemic autoimmunity (205, 216, 231). Thus, we examine whether the Tim-1 mucin domain regulates mast cell activity, particularly in the context of Tim-4 treatment. Using a mutant mouse lacking only the Tim-1 mucin domain (Tim-1^{Δmucin}) (205), we determine whether the Tim-1 mucin domain is necessary for mast cell responses and if Tim-1 mucin domain is required to relay the co-stimulatory effects of Tim-4 binding.

14.2 MATERIALS AND METHODS

14.2.1 Mice

Mutant mice lacking Tim-1 mucin domain (Tim-1^{Δmucin}) were obtained from David Rothstein (University of Pittsburgh), and were originally from Vijay Kuchroo (Harvard Medical School). Age- and sex-matched wild-type C57BL/6 were purchased from the Jackson Laboratory (Bar Harbor, ME) as control. All studies were performed in accordance with University of Pittsburgh Institutional Animal Care and Use Committee procedures.

14.2.2 BMMC isolation and culture

Bone marrow cells from C57BL/6 Tim-1 wild-type (WT) and mutant (Tim-1^{Δmucin}) were cultured in RPMI 1640 supplemented with 10% BGS, non-essential amino acid, 2-ME, HEPES, pen/strep-glutamine, and 20% IL-3-conditioned media for 4-6 weeks, after which >90% of viable cells are mature mast cells (c-kit⁺ FcεRI⁺ by flow cytometry).

14.2.3 Antibodies and reagents

Monoclonal anti-dinitrophenyl (DNP), IgE isotype, clone SPE-7, DNP₃₂-HSA, and 4-Nitrophenyl N-acetyl-β-D-glucosaminide (pNAG) were purchased from Sigma-Aldrich (St. Louis, MO). Purified Tim4-Fc were obtained from Vijay Kuchroo (Harvard Medical School). Human IgG as isotype control for Tim4-Fc was purchased from Jackson ImmunoResearch Laboratories (West

Grove, PA). c-kit-APC and FcεRI-PE (clone MAR-1) antibodies were from eBioscience (San Diego, CA).

14.2.4 BMMC culture, stimulation, and cytokine measurement

BMMCs were sensitized overnight with 1 µg/ml IgE without IL-3 conditioned media. Cells were stimulated with DNP₃₂-HSA or DNP₅-BSA for indicated times. Six hours post stimulation, supernatants were collected and assayed for murine IL-6 and TNF-α by ELISA (BioLegend).

14.2.5 Degranulation assay by flow cytometry

Degranulation was measured as described previously (363). Briefly, BMMCs were loaded with 0.1 µM of LysoTracker Deep Red (Invitrogen) for thirty minutes at 37°C prior to sensitization with 0.5 µg/ml IgE for 1 hour. Ninety minutes post antigen cross-linking by DNP₃₂-HSA, cells were collected and stained with Annexin V (BioLegend). %degranulation was determined as percentage of BMMCs that is AnnexinV⁺LysoTracker^{lo}.

14.2.6 Statistical analysis

All statistical analysis was performed using Prism software (GraphPad Software). Paired, two-tailed Student's t-test, one-way and two-way ANOVA with Bonferroni post hoc test were used for data analysis and determination of p values, as appropriate.

14.3 RESULTS

14.3.1 Tim-1 mucin domain is not required for normal degranulation, cytokine production or Tim-4-mediated co-stimulation in mast cells

We noted no obvious defects in mast cell development or maturation, when BMMC were generated from Tim-1^{Δmucin} bone marrow. Thus, WT and Tim-1^{Δmucin} BMMCs expressed comparable levels of surface Tim-1, based on staining with IgV-binding Tim-1 antibodies (Fig. 14-1A). Tim-1^{Δmucin} BMMCs exhibited a similar extent of degranulation in response to antigen stimulation, compared to WT BMMCs, which was not altered by Tim-1 ligation (Fig. 14-1B-C). Next, we examined the ability of Tim-1^{Δmucin} BMMCs to secrete cytokines in response to IgE/Ag. Tim-1^{Δmucin} mast cells appeared to respond normally to antigen stimulation, and also to Tim4-mediated co-stimulation (Fig. 14-2A). Using different batches of BMMCs over the course of multiple experiments, we were not able to consistently detect any major difference between WT and Tim-1^{Δmucin} BMMCs. We did observe some variation in the amount of IL-6 produced by different batches of BMMCs, but this appeared to be largely due to the relative maturation status of the cells (Fig. 14-2B-E). These results indicated that the mucin domain of Tim-1 is not required for normal mast cell responses and also that Tim-4-mediated mast cell activation does not appear to involve the mucin domain of Tim-1, but rather its IgV domain.

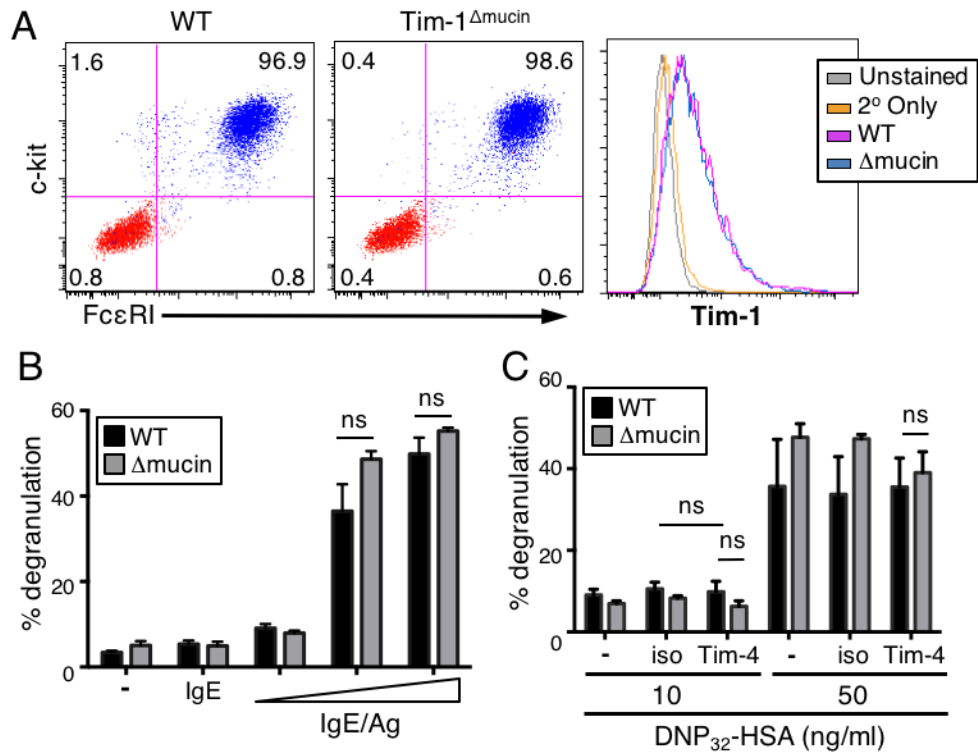


Figure 14-1: Tim-1 mucin domain is not required for mast cell development and degranulation. Tim-1 surface expression and maturity of WT and Tim-1 Δ mucin BMMCs were determined by FcεRI and c-kit staining (A). WT and Tim-1 Δ mucin BMMCs were sensitized overnight with IgE and stimulated with indicated amount of antigen and Tim-4-Fc. Mast cell degranulation was measured by Annexin V and LysoTracker staining (B-C). Results are average of three independent experiments performed in duplicate.

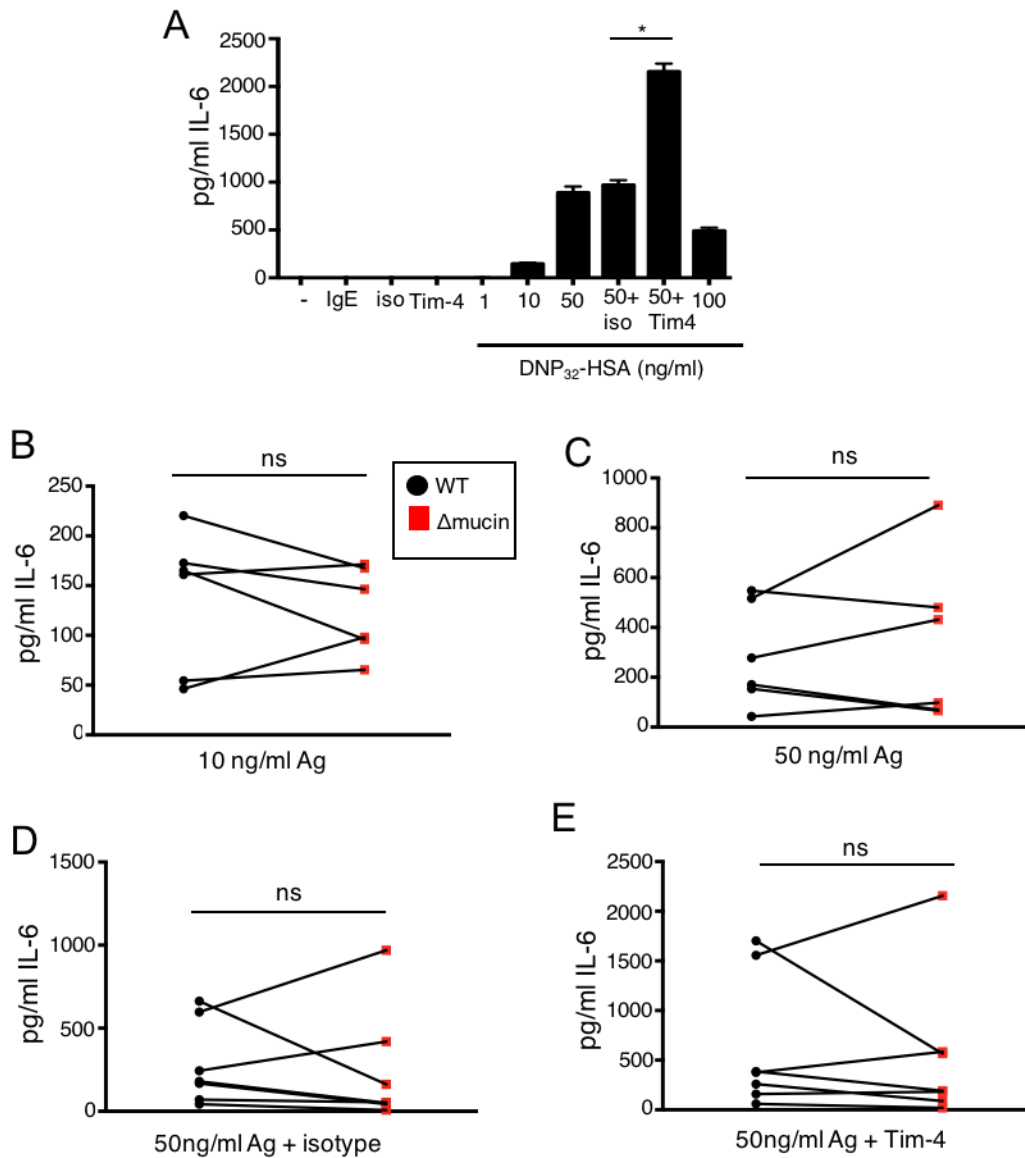


Figure 14-2: Tim-1 mucin domain is not required for Tim4-mediated enhancement of cytokine production in IgE/Ag-stimulated Tim-1^{Δmucin} BMMCs. WT and Tim-1^{Δmucin} BMMCs were sensitized overnight with IgE and stimulated with indicated amount of antigen and Tim4-Fc. Culture supernatants were collected and analyzed for IL-6 by ELISA (A). WT and Tim-1^{Δmucin} BMMCs were stimulated with indicated amount of antigen with either isotype control Fc (iso) or Tim4-Fc for six hours (B-E). Results are representative of six independent experiments from six separate batches of BMMCs.

14.4 DISCUSSION

To determine whether Tim-1 plays a positive or negative regulatory role in mast cells, we first attempted siRNA-mediated knockdown of Tim-1 protein in BMMCs but were unsuccessful in obtaining efficient Tim-1 reduction (data not shown). Recent studies reveal that the Tim-1 mucin domain is important for both T cell activation and effector function as well as regulatory B cell activity (202, 205, 216, 231, 365). Thus, we examined whether the Tim-1 mucin domain regulates mast cell activity, particularly in the context of Tim-4 treatment. Contrary to the effects seen in B and T cells, the Tim-1 mucin domain is dispensable for mast cell activity, as mast cell degranulation and cytokine release remain intact in the absence of the mucin domain. It is worth noting that Tim-1^{Δmucin} BMMCs were actually able to secrete more cytokine than WT BMMCs in some instances. However, after testing six different batches of WT and Tim-1^{Δmucin} BMMCs, it appeared that any differences observed were due to the maturation status of BMMCs and their FcεRI surface expression, rather than any direct effect of deletion of the Tim-1 mucin domain.

While our study focused on bone marrow-derived mast cells, absence of the Tim-1 mucin domain may nonetheless affect trafficking and/or differentiation of other mast cell types in their respective tissue microenvironments *in vivo*. Tim1-Tim4 interaction is thought to occur mostly through the IgV domains of the respective proteins, although there is evidence that Tim-4 may also bind to the Tim-1 mucin domain (195). We showed that Tim-4 mediated co-stimulation of mast cell function occurred independent of the mucin domain. It remains to be determined, in the absence of the Tim-1 mucin domain, whether Tim-4 has other unknown ligands on mast cells that can mediate this enhancement.

15.0 TIM-1: TIM-4 INTERACTION ENHANCES DISTAL RATHER THAN PROXIMAL ANTIGEN RECEPTOR SIGNALING

15.1 INTRODUCTION

We assessed the potential signaling pathways utilized by Tim1-Tim4 interaction downstream of FcεRI signaling to upregulate mast cell cytokine production. Using a Nur77^{GFP} reporter mouse, we previously showed that unlike the related family member Tim-3, engagement of Tim-1 did not enhance FcεRI signal intensity, thereby showing that Tim-1 cross-linking does not augment antigen receptor-proximal signaling (363). We moved on to explore signaling pathways both proximal and distal to the FcεRI complex, for any effects of Tim-1. Thus, the Zap70-related kinase Syk is an FcεRIγ-associated activator integral to activation of LAT, SLP76, PLCγ and other adaptor molecules essential for signal transduction downstream of FcεRI (369). Activation of the “primary” Lyn/Syk/SLP76/PLCγ and the “complementary” Fyn/Gab2/PI3K pathways upon Ag stimulation induces calcium mobilization necessary for degranulation, promotes activity of the MAPK kinases p38, ERK, JNK, and Akt, leading to NF-AT, NF-κB and AP-1 transcriptional activation and subsequent cytokine production (351, 369). Here, we determine the signaling pathways by which Tim-1 ligation enhance IgE/Ag-mediated mast cell activation. We show that Tim-1-mediated IgE/Ag-induced cytokine production is partly due to sustained ribosomal S6 phosphorylation.

15.2 MATERIALS AND METHODS

15.2.1 BMMC isolation and culture

Bone marrow cells isolated from C57BL/6 WT mice were maintained in RPMI 1640 supplemented with 10% BGS, non-essential amino acid, 2-ME, HEPES, pen/strep-glutamine, and 20% IL-3-conditioned media for 4-6 weeks, after which >90% of viable cells are mature mast cells (c-kit⁺ FcεRI⁺ by flow cytometry).

15.2.2 Antibodies and reagents

Monoclonal anti-dinitrophenyl (DNP), IgE isotype, clone SPE-7, and DNP₃₂-HSA were purchased from Sigma-Aldrich (St. Louis, MO). Purified Tim4-Fc were obtained from Vijay Kuchroo (Harvard Medical School) and human IgG as isotype control for Tim4-Fc was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Phospho-specific antibodies to Akt (S374), Syk (Y519/520), S6 (S235/236), p38 (T180/Y182), and SAPK/JNK (T183/Y185) were obtained from Cell Signaling Technology (Danvers, MA). Phospho-specific antibody to ERK (T202/Y204) was from BD Biosciences (San Jose, CA).

15.2.3 BMMC stimulation and western blotting

BMMCs were sensitized with 0.5 µg/ml IgE overnight at 37°C in Tyrode's buffer. Cells were then stimulated with DNP₃₂-HSA for indicated times in Tyrode's buffer. Cells were lysed in 1% Nonidet-P-40 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) supplemented with AEBSF,

aprotinin, leupeptin, pepstatin, sodium fluoride, sodium orthovanadate, and beta-glycerophosphate on ice for twenty minutes. Lysates were centrifuged at 4°C for fifteen minutes. For western blotting analysis, SDS-containing sample buffer was added to lysates prior to loading onto 10% SDS-PAGE gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using the PierceG2 fast blotter (Pierce), probed with appropriate antibodies, and imaged on a ProteinSimple Fluochem M cooled CCD imager (ProteinSimple).

15.2.4 BMMC stimulation and phospho-flow cytometry

BMMCs were sensitized overnight with 1 µg/ml IgE without IL-3 conditioned media. Cells were stimulated with DNP₃₂-HSA for indicated times. Stimulated cells were fixed in 1.5% paraformaldehyde and permeabilized with ice cold methanol. Incubation with phospho-specific antibodies were performed per manufacturer's instructions. Flow acquisition was performed on Fortessa or LSRII (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

15.2.5 Statistical analysis

All statistical analysis was performed using Prism software (GraphPad Software). Paired, two-tailed Student's t-test, one-way and two-way ANOVA with Bonferroni post hoc test were used for data analysis and determination of p values, as appropriate.

15.3 RESULTS

15.3.1 Tim-1 enhances Ag-activated phosphorylation of ribosomal protein S6 without altering FcεRI proximal signaling

We first determined if Tim-4 treatment could modulate the general state of mast cell activation by assessing the total tyrosine phosphorylation in IgE/Ag-stimulated BMMCs in the presence of Tim4-Fc or Tim-3 pAb to cross-linking Tim-3, a co-stimulator of antigen-mediated mast cell responses (363). Thus, Tim4-Fc was not able to enhance tyrosine phosphorylation in mast cells compared to Ag stimulation alone or Ag with Tim-3 pAb co-stimulation (Fig. 15-1). Next, we specifically assessed the Zap70-related kinase Syk, an important initiator at the FcεRI proximal level. Similarly, phosphorylation of Syk was not further increased by Tim-4 (Fig. 15-2A). While we observed robust phosphorylation of ERK and Akt upon IgE/Ag-induced activation, Tim-1 engagement did not yield additional effects (Fig. 15-2B-C). These results demonstrated that MEK/ERK and PI3K/Akt pathways are not involved in enhancement of Ag-mediated mast cell function by Tim-1, even though we observed that phosphorylation of Tim-1 cytoplasmic tail by TCR activation led to recruitment of p85 subunit of PI3K (242). Nevertheless, we did detect a significant increase in phosphorylation of ribosomal protein S6, an important target of the PI3K/mTOR pathway regulating cell growth, survival, metabolism, and protein synthesis in mast cells (361). Mast cells exhibited robust phosphorylation of S6 (~80% of BMMCs) one hour post-Ag stimulation, which did not increase further with Tim-4 addition. However, Tim-4 treatment was able to maintain a significant percentage of pS6-positive BMMCs for as long as four hours, even as the Ag-triggered signal returned to basal levels (Fig. 15-3A). Similar to our previous findings, Tim-1 engagement did not augment Ag receptor signaling as measured by Nur77^{GFP}

expression (Fig. 15-3B and (363)). Overall, these results support a positive role of Tim-1 activation by Tim-4 to sustain mTOR-dependent mast cell metabolism and protein synthesis, leading to enhanced cytokine production.

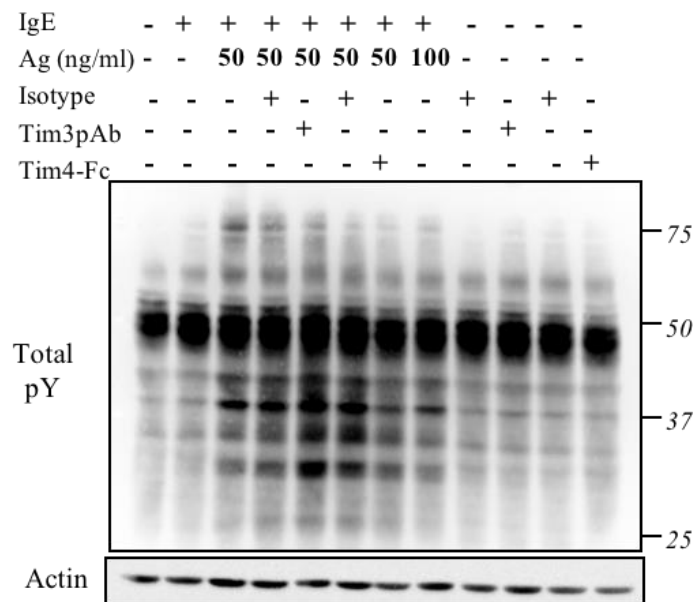


Figure 15-1: Tim-1 engagement did not enhance IgE/Ag-mediate total tyrosine phosphorylation. BMMCs were sensitized with IgE and stimulated with indicated amount of antigen, with 5 μ g/ml of either isotype control, Tim-3 pAb, or Tim4-Fc for ten minutes. Cells were lysed and analyzed for total tyrosine phosphorylation by western blotting. Results are representative of two independent experiments.

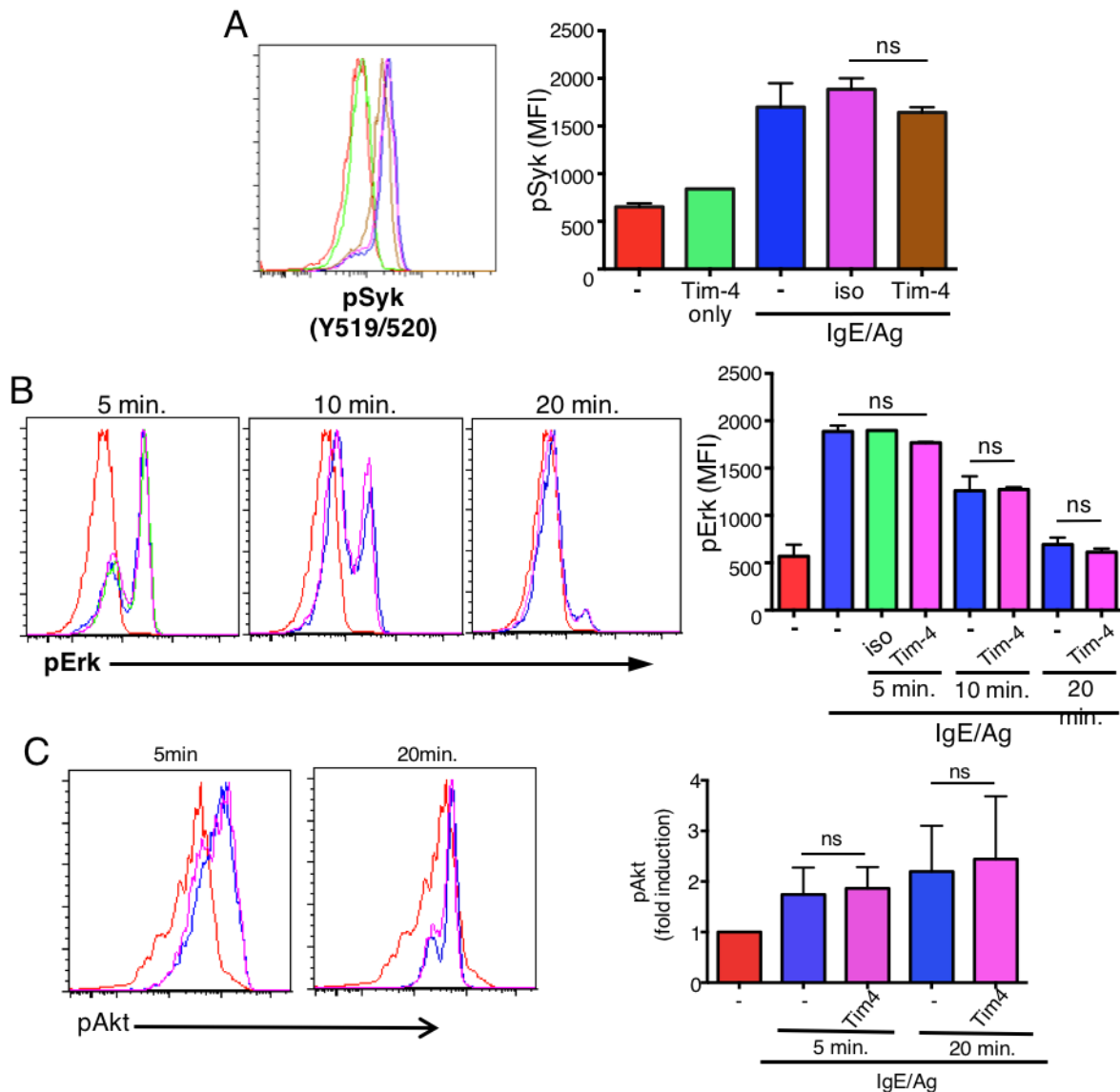


Figure 15-2: Tim-1 ligation by Tim4-Fc did not alter IgE/Ag-mediate Syk, ERK, and Akt phosphorylation. BL/6 BMMCs were sensitized with IgE and stimulated with DNP₃₂-HSA in the presence of isotype control or Tim4-Fc for the indicated time. Syk phosphorylation (Y519/520) (A), pErk (T202/Y204) (B), and pAkt (S374) (C) were analyzed by phospho-flow cytometry. Results are representative of two independent experiments (A-B) and average of two independent experiments (C).

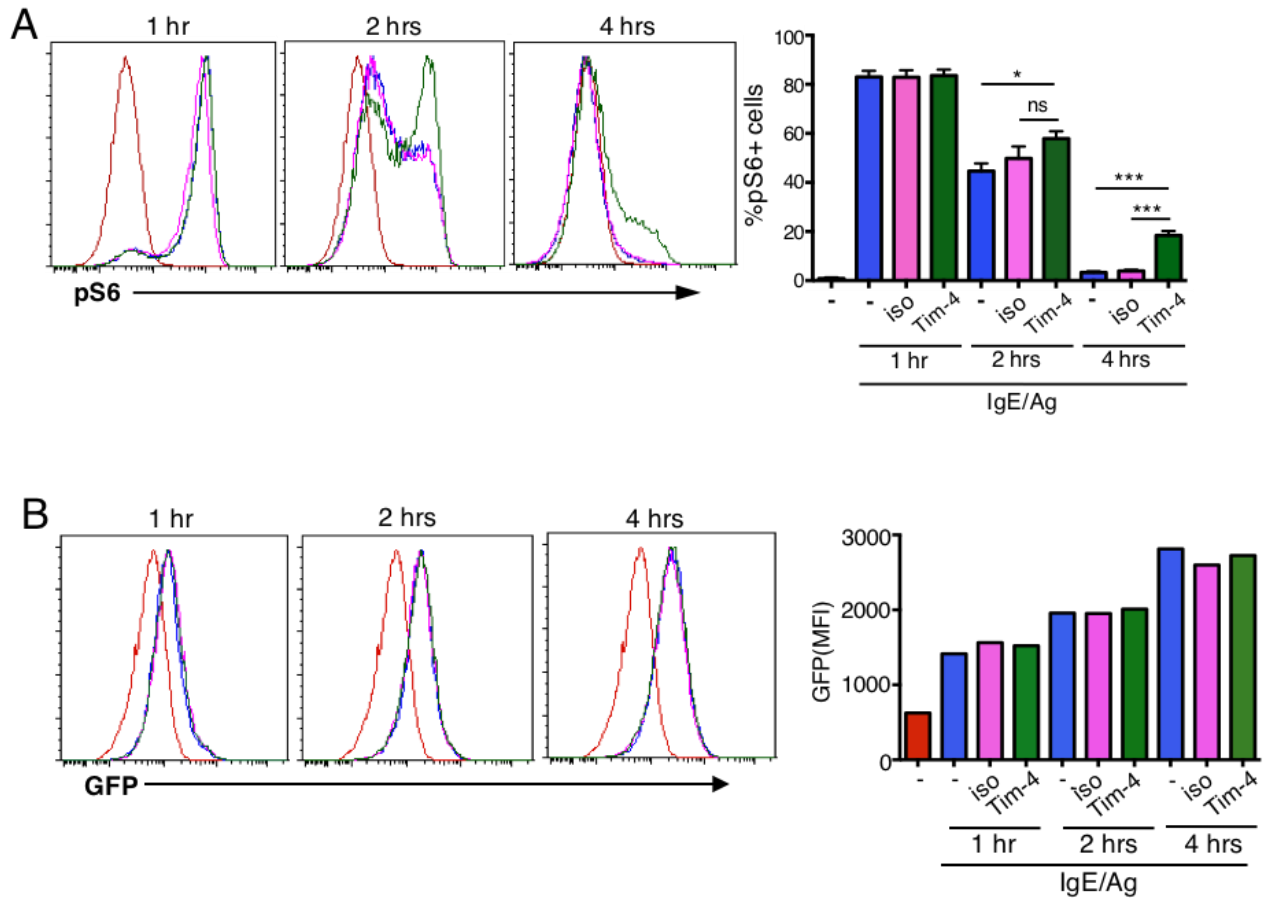


Figure 15-3: Tim-1 ligation by Tim4-Fc enhances mast cell cytokine production by sustaining ribosomal protein S6 phosphorylation upon IgE/Ag activation. BL/6 BMMCs were sensitized with IgE and stimulated with DNP₃₂-HSA in the presence of isotype control or Tim4-Fc for the indicated time. pS6 (S235/236) was analyzed by phospho-flow cytometry. Results are average of three independent experiments (A). Nur77^{GFP} BMMCs were stimulated as indicated prior to GFP quantification by flow cytometry (B). Results are representative two independent experiments.

15.4 DISCUSSION

Here, we focused on examining the signaling pathways leading to enhanced transcriptional activation and cytokine production by Tim-1 and Ag co-stimulation in mast cells. Phosphorylation of Syk was not altered by Tim-4 treatment at the time points we examined. This is consistent with our finding that the Tim1-Tim4 interaction did not promote Ag-stimulated FcεRI signaling intensity, using mast cells from a Nur77^{GFP} mouse model (363). Syk is phosphorylated on multiple tyrosines by either auto-phosphorylation or trans-phosphorylation by Lyn, resulting in positive or negative regulation, respectively, of Ag-mediated FcRI signaling (131, 134, 136). Since we only examined tyrosines 519 and 520 in the kinase loop, which are sites of Syk auto-phosphorylation, it is possible that other tyrosine phosphorylation sites may be affected by Tim-1. One particular site of interest is tyrosine 346 in the linker region, which is important for PLC-γ1 binding and phosphorylation (155). Aside from positive signaling pathways, the relevant negative signaling pathways should be investigated for potential downregulation by Tim-1 engagement. Thus, a particular FcεRI proximal phosphatase of interest is the Src homology-2-containing signaling protein (SHP)-2, which has been implicated in regulation of IgE/Ag-induced IL-6 production through inhibition of NF-κB activity, both of which are enhanced by Tim-1 cross-linking in our study (370).

In a previous study, Tim-4/CD3/CD28-coated beads enhanced phosphorylation of Erk and Akt in CD4 T cells (224). These results were not observed in previously study using both BMMCs and peritoneal mast cells (PMCs) (240). Similarly, we did not observe similar effect with soluble Tim-4 addition in Ag-mediated FcεRI aggregation at the peak of antigen stimulation or when signals returned to basal. It is possible that Tim-4 signals are cell-type specific or that mast cells

require more robust aggregation of Tim-1 at the time of antigen stimulation to induce substantial effects. While phosphorylation of Akt was not affected, ribosomal protein S6 was significantly enhanced upon Tim-4 treatment. Specifically, Tim-1 cross-linking enhanced and sustained IgE/Ag-induced S6 phosphorylation, which correlates with enhanced cytokine production. Ribosomal protein S6 is a downstream effector of PI3K/mTOR signaling, and as such is essential for mast cell survival, proliferation, metabolism and protein synthesis. Consequently, further studies are needed to address whether Tim1-Tim4 interaction promotes mast cell metabolic responses and protein synthesis as well as whether the specific Tim-1 targets in this pathway leading to effector functions.

16.0 SUMMARY AND DISCUSSION

IgE binding to the high affinity receptor FcεRI, and subsequent aggregation by allergens, initiates the assembly of multi-molecular complexes leading to degranulation, transcriptional activation and cytokine production (351, 369). The state of mast cell activation depends on signal integration from both the Lyn/Syk/LAT/PLCγ “primary” pathway and the Fyn/Gab2/PI3K “complementary” pathway. Since Lyn, and to a lesser extent Fyn, can mediate both positive and negative signals upon Ag encounter, it is critical to define the precise molecular mechanisms discriminating activating and inhibitory signals (109, 113, 119, 121). Here, we have identified a co-stimulatory role for Tim-1 and Tim-3 in FcεRI-mediated mast cell activation. Our findings are among the first to describe functions of the Tim family proteins in regulation of mast cell activation aside from their well-known roles in other innate and adaptive immune cell types including T cells, B cells, NK, DC and macrophages.

16.1 TIM-3 REGULATES POSITIVE AND NEGATIVE ANTIGEN SIGNALING

Contrary to the negative regulatory role that has been described in other innate and adaptive immune cells, Tim-3 activity in mast cells is unequivocally positive. This is despite the presence on mast cells of multiple Tim-3 ligands, which have been previously described to mediate inhibition of cellular activation through Tim-3. Our findings suggest that Tim-3 promotes mast cell activation and cytokine production by closely associating with FcεRI and its proximal

signaling pathways. Specifically, our data reveal an active involvement of Tim-3 in controlling the positive and negative effects of Lyn kinase activity. Rather than using Lyn-deficient mast cells that have been associated with enhanced, similar, or reduced degranulation (105, 118, 119, 145), we employed siRNA-mediated Lyn knockdown to mimic the phenotype of *WeeB* mast cells with reduced Lyn catalytic activity (340). In doing so, our findings demonstrated that Tim-3 can either promote positive Ag signaling or actively suppress the negative signals that are normally engaged by stimulation with high Ag concentration or valency. The intensity of Lyn phosphorylation, and its target FcεRIβ, is strictly controlled by the intensity of FcεRI stimulation (109). Furthermore, under conditions of strong Ag stimulation, Lyn is responsible for recruitment to FcεRIβ of the phosphatases SHIP-1 and SHP-1, which carry out a negative feedback suppressive function (109). Therefore, we hypothesize that Tim-3 ligation can counteract the Lyn-dependent negative regulation brought on by high Ag concentration by interfering with SHIP-1 or SHP-1 association with FcεRIβ. Our evidence of a close association between Tim-3 and FcεRI subunits further supports this idea.

Similar to the Tim-1: Tim-4 interaction, Tim-3 ligation sustains Ag-induced ribosomal S6 phosphorylation. Tim-3 pAb could prevent mast cell apoptosis after IL-3 withdrawal, by promoting IL-3 secretion in BMDCs (240). The mTOR kinase is responsible for integrating environmental and intracellular cues such as nutrients, stress, energy and growth factors into diverse biological processes including growth, survival and metabolism (371). Thus, our results imply that Tim-3 engagement may participate in the mTOR signaling pathway to promote mast cell survival, proliferation and protein synthesis. The tuberous sclerosis complex 1 (TSC1) has been reported as a negative regulator of FcεRI-dependent mast cell cytokine production *in vitro* and *in vivo* (372). TSC is also critical for mast cell survival by mediating downregulation of p53

and upregulation of Bcl-2 (372). Therefore, it would be interesting to test whether Tim-3 engagement can enhance mTOR signaling in the absence of TSC1.

mTOR exists as two complexes: mTORC1, which phosphorylates ribosomal S6 kinase and eIF4E-binding proteins (4E-BPs) to promote protein translation; and mTORC2, which phosphorylates Akt, which in turn further activates mTORC1 (371). FcεRI-dependent stimulation of mast cells induces both mTORC1 and mTORC2 activation (178, 361). It is now known that T cells depend on the metabolic regulator mTOR to maintain metabolic programming essential for differentiation and effector responses (373, 374). In T cells, Akt and mTOR support effector T cell differentiation, growth and function by promoting aerobic glycolysis (374, 375). In the periphery, mature naïve T cells mainly oxidize glucose-derived pyruvate in their mitochondria in a process called oxidative phosphorylation (OXPHOS), or fatty acid oxidation (FAO), to generate ATP (373). TCR ligation and engagement of co-stimulatory molecules induces metabolic changes in naïve T cells, to promote more aerobic glycolysis and anabolic biomass accumulation. Thus, in this process, glucose is converted to lactate to generate the metabolic intermediates important for cell growth and proliferation (373). mTOR has also been shown to regulate metabolism in innate cells via similar mechanisms (376). Mitochondrial OXPHOS has recently been shown to participate in ATP-dependent exocytosis in mast cells (377). Nevertheless, the precise mechanisms and implications of mTOR-dependent metabolism in mast cells are virtually unknown. Our findings show that Tim-3 cross-linking promotes Ag-dependent mast cell signaling and cytokine production, all of which could potentially be mediated by mTOR. As a result, we propose that IgE/Ag and Tim-3 pAb co-stimulation can modulate the balance between OXPHOS and aerobic glycolysis as mast cells shift from resting to an activated state. It would be interesting to determine

the metabolic profile of mast cells, i.e. OXPHOS vs. aerobic glycolysis, in the resting state and upon Ag stimulation, and whether Tim-3 ligation can modulate these activities.

Finally, to extend our findings into a clinically relevant setting, we propose to examine human nasal polyp tissue. Thus mast cells have been shown to reside in this tissue and can be activated in an IgE-dependent manner to secrete histamine, leukotrienes and prostaglandin D₂ (PGD₂) (378). In addition, nasal polyps are found in patients with chronic rhinosinusitis (CR) and aspirin-exacerbated respiratory disease (AERD), a severe eosinophilic inflammatory disease of the airways that is also marked by mast cell activation (379), (380). Upon stimulation with anti-IgE, nasal polyp tissue from patients with chronic rhinosinusitis produced more IL-5, IL-2, IL-10, IL-17 and PGD₂ compared to non-atopic controls in a population in Western China (379). In addition, mast cells are among the major IL-25-secreting cells in the nasal polyps of CR patients and polyposis, mucosal edema thickness, and inflammatory cell infiltration (381). AERD patients have increased epithelial expression of IL-33, which has been shown to drive mast cell activation contributing to AERD pathogenesis (380). Tim-3 cross-linking can also enhance Ag-mediated gene expression of IL-2, IL-10 and IL-33 (324). Furthermore, the precise contribution of mast cells in nasal polyp generation is not known and AERD also occurs in non-atopic patients with elevated serum IgE. Therefore, it will be important to determine if Tim-3 is expressed by mast cells in nasal polyp tissue and whether its expression correlates with onset or severity of allergic diseases (382, 383).

Mast cells are a central effector of allergic rhinitis and other inflammatory diseases. Aside from their well-known role in hypersensitivity, mast cell involvement in inflammation-associated with cancer and other non-allergic diseases has been increasingly appreciated (384). Tim-3 is detected on tumor infiltrating human mast cells and its expression is increased upon TGFβ-

treatment (324). Increased numbers of Tim-3/chymase double-positive mast cells have been shown to correlate with the severity of chronic inflammatory periodontitis (330). Manipulation of Tim-3 activity on mast cells could be a promising target for development of novel therapeutic modalities to combat cancer and allergic and autoimmune disease.

16.2 TIM-1:TIM-4 INTERACTION ENHANCES MAST CELL ACTIVATION

The association between atopy and Tim-1 polymorphisms, particularly in the mucin region, have prompted studies to elucidate the mechanisms of action by Tim-1 in allergic diseases (385). More importantly, antibodies to Tim-1 have demonstrated that Tim-1 is a co-stimulatory molecule on T cells, thus raising the possibility of targeting Tim-1 to control allergic inflammation (220, 386). Similarly, Tim-1 has also been identified as a positive regulator of mast cell function (240). Given the genetic and functional connection of Tim-1 to allergy and hypersensitivity as well as the sentinel role of mast cells in atopy, it will be important to determine how Tim-1 signaling contributes to the high affinity Fc receptor for IgE (Fc ϵ RI)-mediated mast cell activation.

Taken together, our findings provide further evidence that Tim-1 signaling can promote cytokine production in Fc ϵ RI-triggered mast cell activation, as well as mechanistic data for how this occurs. Specifically, Tim-1 promotes NF- κ B and NFAT/AP1 transcriptional activation, leading to enhanced IL-6 promoter activation and cytokine production in IgE/Ag-stimulated mast cells. This is in line with a co-stimulatory role for Tim-1 in T, B and NKT cells, both *in vitro* and *in vivo* (206, 216, 231). Contrary to their previously described effects on these cell types, Tim-1 antibodies did not regulate mast cell degranulation and cytokine production in our hands. Nevertheless, Tim-1

ligation by Tim-4 consistently enhanced mast cell cytokine production, and this effect was not affected by loss of the Tim-1 mucin domain. We also showed, for the first time, that unlike in regulatory B cells, the Tim-1 mucin domain is dispensable for mast cell effector function. Finally, we showed that Tim-1, in contrast with Tim-3, acts more distal to FcεRI to enhance S6 activation, without affecting proximal FcεRI signaling. Overall, our findings provide a mechanistic explanation for the co-stimulatory effects of Tim-1 signaling on FcεRI-mediated mast cell activation.

Future studies will focus on identifying the precise mechanisms of Tim-1 cytoplasmic tail phosphorylation, the critical mediator of Tim-1 function, and the signaling complexes recruited by Tim-1 to transduce this positive signal. Our previous findings in T cells showed that Tim-1 can be phosphorylated in an Lck-dependent manner, and can recruit the p85 subunit of PI3K (242). Given other evidence connecting Tim-1 to the PI3K pathway, and the finding that Fyn can phosphorylate the adaptor Gab2 leading to PI3K binding via its SH2-containing domain, we hypothesize that Fyn may phosphorylate Tim-1 to enhance PI3K recruitment (113, 387). Therefore, assessing whether p85 can interact with the Tim-1 cytoplasmic tail in mast cells may reveal a mechanism for the tyrosine phosphorylation-dependent function of Tim-1.

In mast cells, Akt-mediated NF-κB activation and downstream cytokine production require PI3K activity (178). Since Tim-1 expression can augment NF-κB activation in mast cells, we expected that Tim-1 cross-linking by Tim-4 would lead to enhanced Akt phosphorylation and subsequently NF-κB activation. However, this does not seem to be the case as we observed no detectable change in Akt activation. However, ribosomal protein S6 phosphorylation was enhanced, which correlated with enhanced cytokine production. Ribosomal protein S6 is a downstream effector of PI3K/mTOR signaling, and as such is essential for mast cell survival,

proliferation, metabolism and protein synthesis. MALT1 and Bcl10, members of the Carma1-MALT1-Bcl10 (CBM) complex, are essential regulators of FcεRI-induced mast cell activation by selectively upregulating NF-κB-dependent cytokine production without affecting degranulation and leukotriene synthesis (388). We previously identified a Carma1-MALT1-dependent activation of mTOR signaling after TCR engagement, leading to phosphorylation of S6 and another mTOR substrate 4E-BP1 (389). Thus, Tim-1 may preferentially engage the MALT1-Bcl10 pathway to modulate mTOR signaling and NF-κB responses without affecting FcεRI-proximal signaling. Consequently, further studies are needed to address whether Tim1-Tim4 interaction promotes mast cell metabolic responses and protein synthesis, as well as whether the specific Tim-1 targets in this pathway leading to effector functions.

Although induction of NF-AT is also augmented by Tim-1, it remains to be determined whether calcium mobilization is modulated upon Tim-1 expression or co-stimulation. The calcium connection is particularly intriguing, since it ties together NF-AT, calcium-binding PKC isoforms such as PKCβ, NF-κB and AP-1 pathways. Specifically, the calcium-binding isoform PKCβ has been shown to mediate degranulation and IL-6 production in Ag-stimulated mast cells (390). In T and B cells, PKC-θ and -β, respectively, act upstream of the MALT1-Bcl10 complex to regulate NF-κB activation (391). In addition, diacylglycerol (DAG) generated by phosphorylated PLCγ1 and PLCγ2 activates PKCβ and PKCε in mast cells, and contributes to Fos and Jun activation (166). Although the precise role of PKCβ in NF-κB activation is unknown, Tim-1 ligation may selectively direct calcium signaling to promote cytokine production, rather than degranulation, via NFAT and PKC-mediated NF-κB and AP-1 activation.

Finally, Tim-1 blockade or cross-linking by known antibodies and ligands has been shown to ameliorate or exacerbate allergic lung inflammation *in vivo*. In our studies, Tim-4 consistently

enhanced Ag-mediated cytokine production in mast cells. Tim-4 binding to mast cells has been shown to occur specifically through Tim-1, since addition of recombinant Tim-1 could interfere with Tim-4 binding to surface Tim-1 (240). In addition, we observe that Tim-4 binding cannot be detected on BMDCs that have lost Tim-1 expression after an extended time in culture (data not shown). These results indicate that the Tim-1: Tim-4 interaction is specific and that manipulation of Tim-1 activity on mast cells, particularly modulation of the Tim1-Tim4 interaction, could be a novel therapeutic target to control allergic and autoimmune disease.

17.0 CONCLUSION

Allergic diseases, including allergic asthma, rhinitis, atopic dermatitis and food allergy, affect approximately 15 percent of the global population and are on the rise (392). The current treatments for allergic diseases includes antihistamines, glucocorticoids (GCs), leukotriene antagonists, mast cell stabilizers, β -agonists and anticholinergic agents (393). However, these drugs are not an ideal solution as they, particularly GCs and antihistamines, can only control symptoms and often cause serious side-effects in patients (394). Consequently, there is a need to identify specific targets and develop more efficacious immunotherapies to treat atopic diseases.

In recent years, monoclonal anti-IgE antibody has shown promise in treating allergic diseases other than allergic asthma, further emphasizing the importance of targeting IgE-mediated cellular activation to effectively control disease severity (395). Mast cells are among the key mediators of disease pathogenesis, as IgE primarily binds to Fc ϵ RI on mast cells and triggers release of a diverse array of inflammatory mediators. Here, we provide evidence for Tim-1 and Tim-3 as regulators of mast cell responses. We show that Tim-1 and Tim-3 can enhance IgE-mediated mast cell activation by augmenting both the immediate degranulation phase and cytokine production phase. There has been tremendous interest in development of antibodies targeting Tim-1 and Tim-3 in allergy, autoimmunity, cancer and transplant tolerance, all of which involve some degrees of mast cell activation. Our findings not only support mast cell Tim-1 and Tim-3 as relevant therapeutic targets but also provide another layer of complexity in the cell type- and context-dependent effects of the Tim family proteins.

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